LABORATORY MANUAL

on

FUNDAMENTAL PRINCIPLES

OF BACTERIOLOGY

BY

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*The quality of the materials used in the
manufacture of this book is governed by
continued postwar shortages*

PREFACE

This manual is composed largely of the laboratory material that was incorporated in the first edition of "Fundamental Principles of Bacteriology." The experiments are based on important fundamental principles and facts which a student should acquire before proceeding to more advanced work in the field.

Some of the old exercises have been discarded and others improved by the incorporation of new procedures or materials. Every exercise has been completely revised and rewritten. A brief introduction is given to each exercise explaining its purpose and value. The various steps in the procedures have been numbered to facilitate reading by the student. Questions are given at the end of each exercise. The student's answers to these questions determine whether or not he has a sound knowledge of the principles involved in the exercises.

Sufficient experimental material has been included to meet the requirements of beginning students in the bacteriology major and of students in the various divisions of agriculture, as well as forestry, home economics, pharmacy, dentistry, sanitary engineering, chemistry, physical education, hygiene, and public health. The number of experiments should prove ample for a one-semester course of six laboratory hours per week. The experiments cover enough ground to give the student a sound foundation before proceeding to more advanced work on the subject. Most laboratory manuals fail to incorporate sufficient exercises, with the result that the instructor is forced to introduce additional procedures. The author has purposely introduced a large number so that the instructor may make a selection if desired.

The names of the organisms used in the manual are those recommended by the Committee on Classification of the Society of American Bacteriologists and published under the title "Bergey's Manual of Determinative Bacteriology," fifth edition, 1939. This system is not endorsed by all bacteriologists but it comes nearer to being a standard classification than any other that has been used before and it is in general use in this country.

Although the manual can be used in connection with any satisfactory textbook, it is written especially to accompany the author's "Fundamental Principles of Bacteriology," second edition, 1943. The textbook is constantly referred to under the various exercises. The experimental procedures parallel for the most part the textbook material. The ques-

tions listed at the end of each exercise may be answered by consulting the textbook.

The author is greatly indebted to his wife for her aid in reading and checking the proof and to those who have made suggestions during the preparation of the manuscript.

A. J. SALLE.

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LIST OF CULTURES

The following organisms are used in the various experimental procedures in this manual. The organisms are arranged alphabetically according to genera. The names used are in accordance with the fifth edition (1939) of "Bergey's Manual of Determinative Bacteriology." The cultures may be obtained from the American Type Culture Collection, Georgetown University School of Medicine, 3900 Reservoir Road, Washington, D. C.

Aerobacter aerogenes
Alcaligenes faecalis
Alcaligenes viscosus
Bacillus aerosporus
Bacillus cereus
Bacillus freudenreichii
Bacillus megatherium
Bacillus subtilis
Bacillus viridulus
Clostridium sporogenes
Corynebacterium diphtheriae
Erwinia phytophthora
Escherichia coli
Escherichia communior
Flavobacterium synxanthum
Klebsiella pneumoniae
Micrococcus cremoris-viscosi
Micrococcus ureae
Proteus vulgaris
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas syncyanea
Pseudomonas ureae
Rhodococcus roseus
Saccharomyces cerevisiae (yeast)
Sarcina lutea
Sarcina ureae
Serratia marcescens
Staphylococcus aureus
Streptococcus faecalis
Streptococcus lactis

GENERAL LABORATORY RULES AND PROCEDURES

Each student will supply the following:

1 laboratory coat or apron.
1 china marking pencil for writing on glass.
1 laboratory notebook.
1 box microscope slides.
1 dozen cover slips, 22 mm., circular or square.
1 box gummed labels for microscope slides.
1 package string tags, to slip over flasks or to fasten on baskets.
6 blotters for blotting slides.
2 towels for drying glassware.
1 large clean handkerchief for cleaning slides.
1 box matches.

Supplies found in desk:

1 microscope, complete with oculars, and low-power, high-power, and oil-immersion objectives.
1 thermometer 0 to 100°C.
2 needle holders, with wire needles.
1 1000-cc. graduate.
1 100-cc. graduate.
1 granite funnel.
1 wire gauze.
2 test-tube blocks.
1 forceps for holding slides.
1 granite spoon.
1 granite bowl.
2 tumblers.
1 double boiler.
1 test-tube support.
1 ring stand.
2 rings, 3 in. and 5 in. in diameter
1 Bunsen burner.
2-ft. rubber tubing for Bunsen burner.
1 block for holding 8 stain bottles.
8 stain bottles.

Supplies not found in desk but furnished from supply room as needed:

Flasks:
    1000-cc. capacity.
    500-cc. capacity.
    250-cc. capacity.
Test tubes.
Basket for test tubes.

10-cc. graduated pipettes.
1-cc. graduated pipettes.
Pipette can.
Petri dishes.
Petri dish can.

Special apparatus to replace or supplement that in the locker may be secured from the supply room as needed.

Laboratory Rules:

1. Hang wraps, coats, hats, etc., on the hooks provided for this purpose. Never lay or hang them on the laboratory desks.

2. Do not leave valuables in coat pockets.

3. Students should enter the laboratory with clean laboratory coats or aprons. Before being sent to the laundry these coats or aprons should be wrapped in paper and sterilized in the autoclave.

4. Food must not be eaten in the laboratory. Learn immediately to keep paper, pencils, fingers, and other objects out of the mouth.

5. Do not drink from laboratory ware.

6. Moisten labels with water, not with the tongue.

7. Observe all possible cleanliness and neatness in the care of apparatus, microscope, and desk.

8. The inoculating needle used in making cultures should be sterilized in the flame before and after laying down. Flame the wire vertically instead of horizontally. If it is covered with viscous material, dry at the side of the flame before sterilizing, to avoid scattering of living material.

9. In case a living culture is spilled, notify the instructor immediately. It should be covered with an efficient disinfectant. The hands should then be disinfected and washed in soap and water.

10. All noninfectious solid wastes, such as cotton, paper, matches, etc., should be placed in the buckets provided for this purpose in the laboratory.

11. All cultures should be placed in the buckets provided for this purpose in the sterilizing rooms.

12. In case of personal accidents, such as cutting or pricking the fingers, or splashing of culture material in the eye, report immediately to the instructor in charge.

13. At the close of work put away all apparatus and wash the top of your desk with disinfectant.

14. Before leaving the laboratory see that the gas and water are turned off.

15. Wash hands thoroughly with soap and water before leaving the laboratory.

16. Smoking will not be tolerated in the laboratory.

17. Prepare a list of media needed for the day before calling at the supply room. Do not request more material than is actually needed for the day's work.

18. All cultures are to be grown in the 37°C. incubator, unless otherwise directed.

19. Keep complete records of each day's work, with drawings, in a standard laboratory notebook.

20. The following points should be borne in mind in the preparation of your notebook:

> a. Write as neatly as possible. All drawings should be made neatly and accurately.
>
> b. It is better to write with ink.
>
> c. Notes should be recorded as briefly as possible but, on the other hand, elaborate sufficiently so that their meaning will be clear to you and to the instructor reviewing the book.
>
> d. In recording results observe the following:
> Write title of experiment.
> Give object of experiment.
> Record results obtained.
> Draw conclusions, if possible.

CLEANING GLASSWARE

New Glassware.—Place glassware such as tubes, Petri dishes, and flasks into a bucket, cover with a 1 per cent solution of trisodium phosphate, and bring to a boil. Remove the glassware from the phosphate solution and rinse in tap water. Immerse in a 1 per cent hydrochloric acid solution to neutralize the alkalinity. Rinse in tap water and finally in distilled water. Allow to drain until thoroughly dry.

Used Glassware.—Sterilize all glassware in the autoclave at 15 lb pressure for 30 min. This is a safe rule to follow regardless of whether the glassware is contaminated with pathogenic or saprophytic organisms. After sterilization, empty the glassware of their contents and immerse into a 1 per cent solution of trisodium phosphate. Boil for a few minutes. Remove the glassware from the phosphate solution and clean with a brush. Rinse thoroughly in tap water. Then immerse in a 1 per cent solution of hydrochloric acid to neutralize the alkalinity. Again rinse in tap water and finally in distilled water. Allow to drain until thoroughly dry.

Empty the contents of the glassware into the waste buckets provided for this purpose. Under no conditions should the contents be em

into the sink as the hot agar will solidify on cooling and may stopper the drain pipes.

New Pipettes.—Place the pipettes in a shallow pan and cover with a 1 per cent solution of trisodium phosphate. Boil vigorously for 10 min. Rinse thoroughly in tap water. Then immerse in a 1 per cent solution of hydrochloric acid to neutralize the alkalinity. Again rinse in tap water and finally in distilled water. Allow to drain until dry.

Used Pipettes.—Place the pipettes in a jar containing 1 per cent Lysol solution or any other efficient germicide and allow to remain for several hours. Drain the pipettes and place in a shallow pan. Cover with a 1 per cent solution of trisodium phosphate and boil vigorously for 10 min. Rinse thoroughly in tap water. Then immerse in a 1 per cent solution of hydrochloric acid to neutralize the alkalinity. Again rinse in tap water and finally in distilled water. Allow to drain until dry.

New Microscope Slides.—Immerse new slides in alcohol containing 3 per cent hydrochloric acid. Allow to remain for several hours. Remove the slides from the acid alcohol, rinse thoroughly in tap water and finally in distilled water. Wipe dry with a soft, clean cloth. Just before use pass the slide a few times through the flame to burn off any grease that may be present. Cool before use. Be sure to use only the flamed side of the slide. Keep the slides in a covered jar or Petri dish.

New Cover Slips.—Drop the cover slips, one by one, into a dish containing acid alcohol and allow to remain for several hours. Pour off the acid alcohol. Rinse the cover slips thoroughly in tap water and finally in distilled water. Wipe dry with a soft, clean cloth. When wiping, hold the cover slips by their edges to prevent recontamination with grease. Keep the cover slips in a covered jar or Petri dish.

Used Slides and Cover Slips.—Place the slides and cover slips in a pan and cover with a 1 per cent solution of trisodium phosphate. Boil vigorously for 20 min. Rinse thoroughly in tap water. Then immerse in a 1 per cent solution of hydrochloric acid to neutralize the alkalinity. Again rinse in tap water and finally in distilled water. Wipe dry with a soft, clean cloth using the same precautions in drying as given under New Cover Slips.

PLUGGING GLASSWARE

All test tubes, pipettes, and flasks must be carefully plugged with cotton before being sterilized. Either absorbent or nonabsorbent cotton may be used. The latter is preferable because it resists wetting by liquids in case test tubes or flasks tip over.

For a test tube use a small piece of cotton about 2 in. square. Strengthen the center of the square with a small additional piece of

cotton.   Place the square over the opening of the test tube and force down into the tube with a glass rod to a depth of about 1 in.

Wrap large plugs for flasks and bottles in cheesecloth to prevent fraying.

Plug pipettes lightly at the mouth end only.   Burn off the excess cotton by passing the pipettes through a flame.   Then place them tip downward into the metal can provided for this purpose.

STERILIZATION BY HEAT

Sterilization by heat may be defined as the complete destruction of all living organisms including their spores.

Plugged test tubes, flasks, pipettes, bottles, etc., must be sterilized before use in order to destroy all living organisms adhering to the inner surfaces of the glassware.   Likewise all culture media must be sterilized previous to use to destroy all contaminating microorganisms present. Studies on single bacterial species or pure cultures could not be made if the glassware and culture media were contaminated with other kinds of organisms previous to use.   When once sterilized, glassware may be kept in a sterile condition indefinitely if protected from outside contamination.   The same applies to culture media if, in addition to sterility, evaporation can be prevented.

The usual procedures employed for the sterilization of glassware, media, cotton, etc., involve the use of heat.   Three general types of heat sterilizers are used in bacteriological laboratories for the destruction of living microorganisms.   These are known as the hot-air sterilizer, the Arnold sterilizer, and the autoclave.

Hot-air Sterilizer.—The hot-air sterilizer is used for sterilizing such glassware as test tubes, pipettes, and Petri dishes.   The autoclave or sterilization by steam under pressure may also be used where speed is desired, but the glassware becomes wetted by the steam and, for some purposes, must be dried before use.

The sterilizer is operated at a temperature of 160 to 180°C. for a period of 1½ hr.   If the temperature goes above 180°C., the cotton stoppers may char, giving the glassware a smoky appearance.   Therefore the thermometer must be watched closely at first until the sterilizer is regulated to the desired temperature.

The Arnold Sterilizer.—In the Arnold, sterilization is effected by streaming steam at a temperature of about 100°C. for 20 min. on three successive days.   The principle underlying this method is that the first sterilization kills all the vegetative organisms (*i.e.*, not spores) present. After a lapse of 24 hr. in a favorable medium the spores, if present, will pass into the vegetative forms.   The second sterilization will again destroy all vegetative organisms.   It sometimes happens that not all

the spores pass into vegetative forms before the second heating. Therefore the process is repeated the third time to make sure all living organisms are destroyed.

It must be remembered that a temperature of 100°C. for 20 min. is not sufficient to destroy spores. A much higher temperature is required to effect a complete sterilization in one operation over a relatively short period of time.

The Arnold is used principally to sterilize gelatin, milk, and carbohydrate media. Higher temperatures or longer single exposures in the Arnold may decompose carbohydrates and prevent gelatin from solidifying. Obviously such media would then be unsatisfactory for use.

The Autoclave.—The autoclave destroys both vegetative cells and spores in one operation. It is usually operated at a temperature of 120°C., for 30 min. This corresponds to a steam pressure of about 15 lb.

The principle of the method is that water boils at about 100°C., depending upon the vapor pressure of the atmosphere. If the atmospheric pressure is increased, the temperature will be likewise increased. Therefore if the steam pressure is increased inside of the closed vessel to 15 lb. (2 atm.), the temperature will rise to 121.6°C. The following table will show the relationship between pressure and temperature:

Pressure, lb.	Temperature	
	°C.	°F.
5	107.7	227
10	115.5	240
15	121.6	250
20	126.6	260
25	130.5	267
30	134.4	274

The autoclave is used to sterilize the usual noncarbohydrate broths and agar media, discarded cultures, contaminated tubes, flasks, dishes, aprons, rubber tubing, rubber stoppers, etc.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE MICROSCOPE

Acquaint yourself with the mechanical construction and names of the various parts of the microscope. Also, read thoroughly the following points on the manipulation and care of the instrument.

THE USE OF THE MICROSCOPE

1. The microscope should be placed on the desk with the two ends of the horseshoe base away from you. The stage should be kept in a horizontal position. This prevents the immersion oil or other liquids from running off of the slide.

2. Bring the draw tube to the standard length for which the objectives are corrected (usually 160 mm.).

3. The exterior lens of the eyepiece should always be carefully cleaned before use by wiping with lens paper. Being exposed, this lens easily accumulates dust from the air.

4. The back lens of the eyepiece may be cleaned by wrapping clean lens paper about the end of a rounded soft wooden stick and wiping with a slight twirling motion. Do not use alcohol as a cleaning fluid.

5. Objective lenses should be kept clean at all times. They are best cleaned by wiping with lens paper. Under no conditions should objectives be removed from the revolving nosepiece unless it is necessary to clean the back lens. For this purpose follow the same procedure as given under (4) above.

6. Dried cedarwood immersion oil may be removed by rubbing the objective lens with a piece of lens paper previously moistened with a drop of xylol. If paraffin (mineral) oil is used for the immersion lens instead of cedarwood oil, the use of a solvent is not necessary.

7. Illumination of the object should be carried out by adjusting the mirror with respect 'to the source of light. This should be very carefully done; otherwise one may easily fail to obtain the best results, may be easily led to wrong conclusions, or may even injure the eyes.

8. If the microscope is equipped with a substage condenser, always use the plane mirror. If used without a condenser, either surface may be employed, the concave mirror yielding more intense illumination.

9. In focusing the low-power objective, first place the slide upon the stage of the microscope under the spring clips. Swing the low-power objective under the body tube and, using the coarse adjustment, lower the objective until the point of it is about ¼ in. from the object. Then,

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while looking through the eyepiece, slowly elevate by the coarse adjustment until the image is distinct. Focus sharply by means of the fine adjustment.
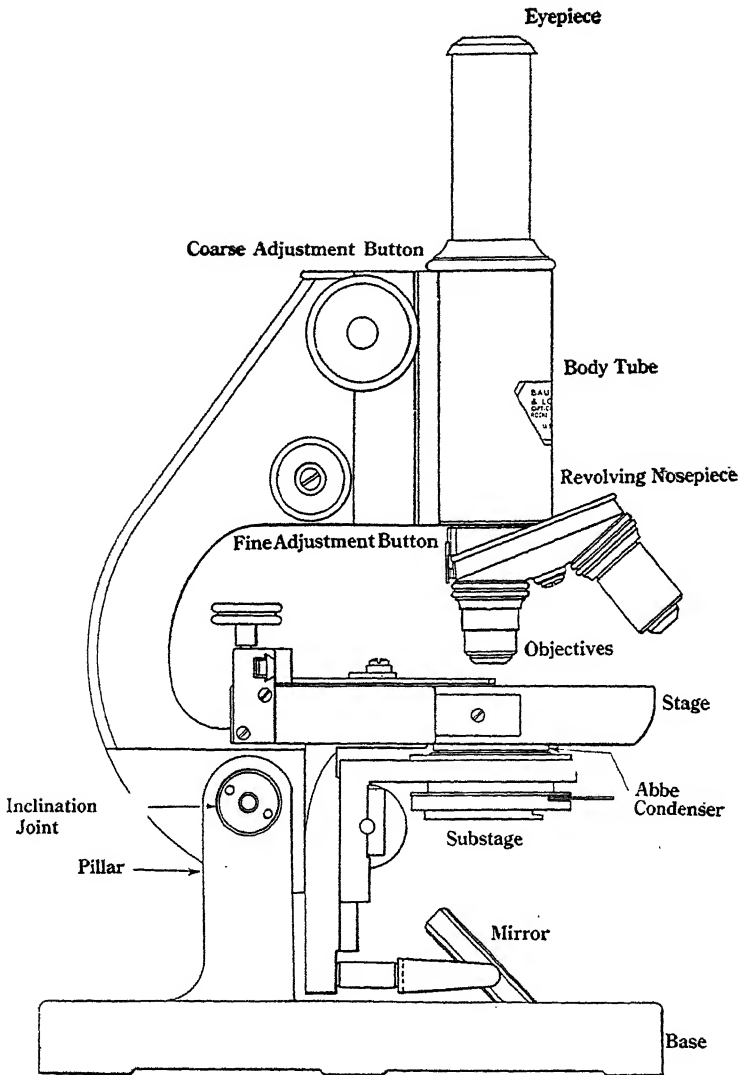
Fig. 1.—The microscope and its parts.

10. In focusing the high-power objective, first swing the lens under the body tube. Then, while looking between the objective and the slide,

slowly lower the lens with the coarse adjustment until the front of the objective almost touches the slide. Focus upward by means of the fine adjustment until the image is in sharp focus.

11. In focusing the oil-immersion objective, first place a drop of oil over the object on the slide. Lower the objective very carefully with the coarse adjustment until contact is made with the glass. Do not lower the objective too rapidly, otherwise there will be danger of cracking the glass slide with possible injury to the objective lens. Then focus upward with the fine adjustment until the image is in sharp focus.

12. The immersion oil should have as near as possible the same refractive index as the glass. Cedarwood oil is the best for this purpose but, since it dries to a hard resin, it is necessary to wipe off the lens at the close of work. To overcome this difficulty paraffin or mineral oil may be substituted for the cedarwood oil. It has the advantage of not drying, thus doing away with the necessity of wiping the lens or using xylol to remove the dried accumulation from the previous period. It has the disadvantage of giving a poorer definition than cedarwood oil owing to its smaller index of refraction.

13. If the fine adjustment fails to function, it has probably been screwed up too far. Screw it down about half the length of the threads before focusing on the object.

14. The substage iris diaphragm serves several purposes. It may be used to control the intensity of the illumination but it is not recommended for this purpose. Neutral filters should be used to reduce the intensity of the light. Too much light will cause an uncomfortable glare; too little will cause undue exertion; both should be avoided. The main functions of the diaphragm are to increase contrast and to improve definition. It happens many times that different diaphragm openings are required for different types of work within the same slide preparation.

15. Never close one eye when using the microscope. This causes eyestrain. Make it a habit to keep both eyes open. This may seem difficult at first but the habit is soon acquired.

THE CARE OF THE MICROSCOPE

1. Keep the microscope free from dust. This rule should be observed at all times.

2. When handling the microscope grasp it by the handle arm, not by the fine-adjustment head or other parts of the instrument.

3. Do not jar or tip over the microscope.

4. Do not use alcohol on the lacquered parts of the instrument as it acts as a solvent. The lacquer prevents oxidation of the metal and should not be removed. Although modern finishes are relatively alcohol

resistant, some of them are nevertheless more soluble in alcohol than in xylol.

5. Keep the coarse adjustment free from dust. If the rack and pinion need lubricating, use a small amount of noncorrosive vaseline. Do not use oil for lubricating a sliding bearing.

6. Do not carelessly turn the coarse adjustment screw to permit violent contact of the front lens of the objective with the glass slide. This may ruin the objective lens.

7. Make it a habit to clean the immersion objective at the close of each period. Use lens paper for wiping.

8. Keep the eyepiece lens clean. If the lens becomes soiled or coated, the image will be indistinct.

EXERCISE 1

MICROSCOPIC EXAMINATION OF HAY INFUSION

Hay infusion may be prepared by mixing a little hay, obtained from an animal cage, with tap water in an open vessel and allowing it to stand for about one week.

Since most bacterial cells contain no chlorophyll and are colorless and transparent, it is very difficult to study them in their natural state. Hanging-drop preparations yield, therefore, only a limited amount of information. To reveal their outline and internal structure they must be stained. The purpose of this exercise is to demonstrate the form, arrangement, size, and motility of a few of the various classes of micro-organisms encountered in nature.

Required:
   1. Hay infusion.
   2. Slides and cover slips.
   3. Two 1-cc. pipettes.

Procedure:
   1. Remove some of the surface liquid of the infusion with a 1-cc. pipette and place 1 drop on a clean glass slide.
   2. Cover with a clean cover slip and place the slide on the stage of the microscope.
   3. First focus with the low-power objective.
   4. After you have focused and examined the slide with the low-power objective, swing the high-power lens into place.
   5. Examine the infusion for bacteria, protozoa, yeasts, molds, and algae. Some or all of these classes of organisms will be present.
   6. Make drawings in your notebook of the various classes of organisms, paying particular attention to their relative size. Also make drawings as accurately as possible.
   7. Repeat the above observations using a drop of the infusion from the sediment.

8. The infusion may be obtained as follows: Hold your finger tightly over the mouth of a sterile 1-cc. pipette. Place the pipette into the infusion and, when the tip reaches the bottom, remove your finger. Again hold your finger over the mouth of the pipette and withdraw it from the infusion.

9. Use only 1 drop on a clean slide.

10. Make observations and drawings as before.

Questions:

1. Does the sediment show the same picture as the scum?

2. Does the quantity of dissolved oxygen play a part in the distribution of the various kinds of organisms in the infusion? Explain.

3. Why is hay, obtained from an animal cage, used in preparing the infusion?

References

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

SMITH, G. M.: "Cryptogamic Botany," New York, McGraw-Hill Book Company, Inc., vol. I, 1938.

THRESH, J. C., and J. F. BEALE: "Examination of Waters and Water Supplies," Philadelphia, The Blakiston Company, 1925.

WARD, H. B., and G. C. WHIPPLE: "Freshwater Biology," New York, John Wiley & Sons, Inc., 1918.

WHIPPLE, G. C., G. M. FAIR, and M. C. WHIPPLE: "The Microscopy of Drinking Water." New York, John Wiley & Sons, Inc., 1927.

EXERCISE 2

CARBOLFUCHSIN STAIN

A diluted solution of carbolfuchsin is very satisfactory as a simpl stain for general bacteriological work.

This exercise demonstrates the method of preparing bacterial smear and staining them by a simple staining technique.

Required:

1. 24-hr. nutrient agar slant culture of *Escherichia coli*.

2. 24-hr. nutrient agar slant culture of *Staphylococcus aureus*.

3. 24-hr. nutrient agar slant culture of *Bacillus subtilis*.

4. Clean slides.

5. Ziehl-Neelsen's carbolfuchsin stain diluted 10 times.

Procedure:

1. Gently heat one side of a clean slide in the flame of a Bunsen burner to remov any grease present.

2. Place the slide on your desk with the flamed side up.

3. Heat the wire loop to destroy any organisms adhering to the surface.

4. Place a loopful of distilled water in the center of the slide.

5. Flame the wire loop before setting it down on the laboratory table.

6. Sterilize the straight inoculating needle in the flame.

7. Remove the cotton stopper from a 24-hr. nutrient agar slant culture of *E. col.* by grasping it with the small finger of the right hand.

8. Flame the neck of the tube and remove a minute amount of growth from the culture.

9. Again flame the neck of the tube, replace the cotton stopper, and set the culture in the test-tube block.

10. Emulsify the growth on the needle in the loopful of distilled water on the slide.

11. Spread the suspension over an area of about ½ sq. in.

12. Again flame the inoculating needle before setting it down on the laboratory table. Make it a rule always to flame the inoculating needle immediately before and after use.

13. Dry the film by holding the slide high over a low gas flame. Do not allow the liquid to steam.

14. Fix the film by quickly passing the slide five or six times through the upper portion of the Bunsen flame. This prevents the film from being washed off during the staining process.

15. In like manner prepare smears from the 24-hr. nutrient agar slant cultures of *S. aureus* and *B. subtilis*.

16. Cover the fixed films with diluted carbolfuchsin stain. Use only sufficient to cover the film, not the entire slide. Practice economy.

17. Allow the stain to remain for ½ min.

18. Wash the slides in tap water, drain, blot, and air-dry.

19. Examine under the oil-immersion objective.

20. Make drawings of the organisms in your laboratory notebook, paying particular attention to their relative sizes.

Questions:

1. How is Ziehl-Neelsen's carbolfuchsin stain prepared?

2. Why is the phenol added?

3. Why is this called a simple stain?

4. Why should the liquid on the slide not be allowed to steam while being dried over a flame?

5. Why is the film fixed?

References

CONN, H. J.: "Biological Stains," Geneva, N. Y., Commission on Standardization of Biological Stains, 1940.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 3

METHYLENE BLUE STAIN

Methylene blue is probably the most widely used dye in bacteriological techniques.

In this exercise it is employed as a simple bacterial staining solution.

Required:

1. 24-hr. nutrient agar slant culture of *Escherichia coli*.

2. 24-hr. nutrient agar slant culture of *Bacillus subtilis*.

3. Methylene blue staining solution.

4. Slides.

Procedure:

1. Follow the same method as given under Exercise 2, but increase the staining time to 3 min.

2. Make drawings of the organisms in your laboratory notebook.

Questions:

1. Does methylene blue stain as intensely as carbolfuchsin?

2. Why is the staining period increased with methylene blue?

3. Why is potassium hydroxide generally added to solutions of methylene blue? Is it still recommended for this purpose?

4. What is "Methylene Blue, Medicinal"?

References

CONN, H. J.: "Biological Stains," Geneva, N. Y., Commission on Standardization of Biological Stains, 1940.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE MORPHOLOGY OF BACTERIA

EXERCISE 4

THE SIZE OF BACTERIA

Several methods are followed for determining the size of bacteria. The procedure usually used consists in unscrewing the upper lens of the eyepiece, inserting an ocular micrometer, and replacing the eyepiece lens. The ocular micrometer is then standardized by means of a stage micrometer. After this has been accomplished, the stage micrometer is removed from the microscope and a stained bacterial preparation inserted in its place.

Required:

1. Stained slides of *Staphylococcus aureus* and *Bacillus subtilis*.
2. Ocular micrometer.
3. Stage micrometer.

Procedure:

Microscopes differ slightly in tube length. This means that their magnifying powers will vary slightly, even though the oculars and objectives are the same. Therefore, it is necessary to standardize each ocular micrometer in terms of a standard stage micrometer.

1. The microscope must always be set to the same tube length.
2. Unscrew the upper lens of the ocular. A metal shelf will be seen inside of the eyepiece.
3. Rest an ocular micrometer on the shelf and replace the upper eyepiece lens.
4. Place a stage micrometer on the stage of the microscope and fasten securely by means of the two removable spring clips.
5. Focus first with the low-power 16-mm. objective.
6. The stage micrometer contains lines exactly 0.01 mm. apart. A distance of 2 mm. is divided into 200 equal parts.
7. Turn the eyepiece until the graduations on the ocular micrometer superimpose and are parallel with those on the stage micrometer.
8. Make sure that the lines at one end coincide.
9. Now look for another line on the ocular micrometer that coincides with one on the stage micrometer.
10. Count the number of lines on the ocular and stage micrometers between the lines that coincide. This gives all the data that are necessary.
11. As an example:

Stage-micrometer rulings: Two millimeters divided into 200 parts. Each division is equal to 0.01 mm.

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Ocular-micrometer rulings: An unknown distance is divided into 100 parts.

55 ocular divisions = 40 objective divisions.
1 ocular division = 0.73 objective division.
1 objective division = 0.01 mm. or 10 μ (microns).
1 ocular division = 0.73 × 0.01 = 0.0073 mm. or 7.3 μ.

12. In the same manner standardize the ocular micrometer for the high-power (4-mm.) and oil-immersion (1.9-mm.) objectives.

13. Now insert the stained slides in place of the stage micrometer and measure the organisms.

14. Record all results in the following table. Show calculations.

Microscope no.		Ocular no.	Tube length, mm.
Value of each ocular division, μ	Low-power objective no.	High-power objective no.	Oil-immersion objective no.

MEASUREMENT OF BACTERIA

Oil-immersion lens	*S. aureus,* diameter, μ	*B. subtilis,* length, μ width, μ

Questions:

1. Why should the eyepiece micrometer be standardized for each microscope?

2. What effect will an increase in tube length have on the value of each ocular-micrometer division?

3. What effect will an increase in magnifying power of the eyepiece have on the value of each ocular-micrometer division?

4. Do bacteria vary in size with age?

5. What age of culture should be employed for making measurements of bacteria?

6. What effect do fixing and staining have on bacterial cell size?

7. What is a micron? A millimicron?

References

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EXERCISE 5

THE MOTILITY OF BACTERIA

Some bacteria are motile and others are not. Two types of motion have been recognized in bacteria: (1) vital movement and (2) Brownian movement.

Independent bacterial motion is due to the presence of organs of locomotion known as flagella (singular, flagellum). Brownian movement

may be described as a quivering or back and forth motion exhibited by very small particles suspended in a liquid.   It is not due to the presence of flagella but to the bombardment of the small particles by the molecules of the suspending fluid.

Motility in bacteria may be conveniently determined by making a hanging-drop preparation from a liquid culture and observing the organisms in the unstained condition.

Required:

1. 18- to 24-hr. nutrient broth culture of *Bacillus subtilis.*
2. 18- to 24-hr. nutrient broth culture of *Staphylococcus aureus.*
3. Clean slides.
4. Clean cover slips.

Procedure:

1. Place two or three loopfuls of an 18- to 24-hr. nutrient broth culture of *B. subtilis* on a clean slide.
2. Flame the inoculating loop each time after a loopful of culture is deposited on the slide; otherwise the culture in the tube may become contaminated by organisms present on the slide.
3. Gently cover the culture with a clean cover slip.
4. Place the slide on the stage of the microscope.
5. Focus with the low-power lens; then swing the high-power objective in place.
6. Determine whether there is true motion or merely Brownian movement.   Do not be confused by streaming movements due to the readjustment of the thickness of the liquid between the slide and the cover slip.
7. Repeat the above experiment using an 18- to 24-hr. nutrient broth culture of *S. aureus.*
8. Record your observations in the following table:

Organism	Brownian movement	Vital movement
B. subtilis		
S. aureus		

Questions:

1. Can you determine from the shape of an organism whether or not it is motile?
2. Is it true that the speed of a motile organism depends upon the number of flagella?
3. Is it true that a motile organism may temporarily lose its power of locomotion?
4. Would you consider this to be the best method for determining the presence or absence of flagella?
5. Can the flagella be seen in a hanging-drop preparation?

References

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EXERCISE 6

INTRAVITAL STAINING OF BACTERIA

The examination of stained smears of dead bacteria does not always give a true morphological picture of their internal structure. If, on the other hand, organisms are stained with a dye solution so highly diluted that it exhibits no toxic action, the structure of bacteria can be studied while the cells are still living.

The protoplasm of young cells appears to be quite homogeneous when stained. The colloidal constituents of the protoplasm appear to be in a high state of division. Granules of any size are very few and difficult to see. The granules increase in size with age, which means that old cells show a very granular appearance. The granules cannot be differentiated from nuclear bodies. The first process that takes place in the reproduction of bacteria is a separation of the cell contents into two polar masses. The cell membrane forms when the cell begins to constrict at the center and at right angles to the polar masses. This is the beginning of cell division and proceeds until the process is complete.

Required:

1. 48-hr. nutrient agar slant culture of *Bacillus subtilis*.
2. 1:5000 aqueous solution of crystal violet.
3. Clean slides.
4. Clean cover slips.

Procedure:

1. Place two loopfuls of a 1:5000 aqueous solution of crystal violet on a slide.
2. Remove a minute amount of the growth from a 24-hr. nutrient agar slant culture of *B. subtilis* and emulsify in the stain solution.
3. Cover lightly with a cover slip.
4. Examine under the oil-immersion objective.
5. Make drawings of the organisms in your laboratory notebook.

Questions:

1. Are the organisms motile?
2. Why do granules appear as the cells become older?
3. What can be said about the chemical composition of the granules?
4. Do the spores take the stain? Why?

Reference

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EXERCISE 7

METACHROMATIC GRANULES IN BACTERIA

Metachromatic granules or volutin take a very deep stain when treated with basic dyes, such as basic fuchsin, crystal violet, or methyl violet. In this respect they behave very much like chromatin but they do not exhibit the characters of nuclear material. As a general rule, granules are more pronounced in old cells after growth has ceased. They are not believed to be living constituents of the cell but stored-up reserve food material of a nitrogenous nature.

The presence of metachromatic granules is made use of in identifying and classifying certain bacteria.

Required:

1. 18- to 24-hr. culture of *Corynebacterium diphtheriae* grown on Löffler's blood serum medium. The organisms are suspended in 0.85 per cent sodium chloride solution containing 0.5 per cent phenol.
2. Albert's diphtheria stain.
3. Neisser's diphtheria stain.
4. Lugol's iodine solution.
5. Slides.

Procedure:

ALBERT'S STAINING METHOD

1. Gently heat one side of a clean slide in the flame of a Bunsen burner to remove any grease present.
2. Place the slide on the table with the flamed side up.
3. Heat the wire loop to destroy any organisms adhering to the surface.
4. Remove the cotton stopper from the saline suspension of *C. diphtheriae* and flame the neck of the tube.
5. Remove one loopful of the bacterial suspension, again flame the neck of the tube, and replace the cotton stopper.
6. Place the loopful of suspension in the center of the flamed slide and spread the liquid over an area about ½ in. square.
7. Flame the wire loop before setting it down on the laboratory table.
8. Dry the film by holding the slide high over a low gas flame. Do not allow the liquid to steam.
9. Fix the film by quickly passing the slide 5 or 6 times through the upper portion of the Bunsen flame. This prevents the film from being washed off during the staining process.
10. Cover the film with Albert's diphtheria stain.
11. Allow the stain to remain for 5 min.
12. Drain the slide without washing.
13. Apply Lugol's iodine solution and allow it to act for 1 min.
14. Wash the slide briefly in tap water, drain, blot, and air-dry.
15. Examine under the oil-immersion objective. The metachromatic granules take a deep blue stain.

Neisser's Double Staining Method

1. Prepare another slide from the saline suspension of *C. diphtheriae* by the same procedure given above.

2. Cover the film with a mixture of 2 parts of solution 1 and 1 part of solution 2. These solutions should be kept separate and mixed only before use.

3. Allow the stain to remain for 10 sec.

4. Wash the slide in tap water.

5. Cover the film with solution 3 and allow it to remain for 10 sec.

6. Wash briefly in tap water, drain, blot, and air-dry.

7. Examine under the oil-immersion lens. The metachromatic granules stain blue while the remainder of the cell takes a brown stain.

Questions:

1. How is Albert's stain prepared?
2. How is Neisser's diphtheria stain prepared?
3. What are metachromatic granules?
4. Why are they usually absent in very young cells?
5. In what organism is the presence of granules of diagnostic importance?
6. Why do metachromatic granules take a deep stain with basic dyes?
7. What other kinds of granules are present in bacterial organisms?

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Exercise 8

THE FLAGELLA OF BACTERIA

Two types of movement are recognized in bacteria: (1) true or vital movement and (2) Brownian movement.

True or vital movement is due to the presence of long, whip-like organs of locomotion named flagella. Brownian movement, on the other hand, is not due to the presence of flagella but may be described as a quivering or back and forth motion produced by the bombardment of the bacteria by the molecules of the suspending fluid.

Staining bacterial flagella is a difficult technique unless certain well-recognized precautions are observed. The most important of these are the following:

1. Slides must be free from grease.

2. Cultures must be young and vigorous; 18- to 22-hr.-old cultures appear to give the best results.

3. Organisms must be cultivated on a satisfactory medium.

4. Organisms must be suspended in distilled water that is not too hot or too cold but having, preferably, the same temperature as that of the laboratory.

5. Organisms must not be allowed to stand too long in contact with the suspending fluid; this varies from 5 to 30 min.—depending upon the organism.

6. Bacterial suspension must be spread out thinly so that the liquid will dry rapidly.

7. A mordant is absolutely necessary for a successful flagella stain.

Required:

1. 18- to 22-hr. nutrient agar slant culture of *Proteus vulgaris*.
2. 2 tubes of distilled water.
3. 2 small funnels.
4. Filter paper.
5. 2 small test tubes with cork stoppers.
6. Gray's mordant.
7. Plimmer and Paine's mordant.
8. Ziehl-Neelsen's carbolfuchsin stain.
9. Saturated alcoholic solution of basic fuchsin.
10. Cleaning solution (sodium dichromate dissolved in sulfuric acid).
11. 95 per cent alcohol.
12. Slides.
13. Pipettes.

Procedure:

CONN AND WOLFE'S METHOD

1. Boil slides in dilute cleaning solution for about 20 min.

2. Rinse thoroughly in tap water. Handle the slides by their edges to avoid redeposition of grease.

3. Immerse the slides in 95 per cent alcohol and allow to remain for about 10 min.

4. Drain the alcohol from several slides, place on a wire gauze, and strongly roast them over a Bunsen burner.

5. Allow the slides to cool to room temperature.

6. Use an 18- to 22-hr. nutrient agar slant culture of *P. vulgaris*, previously transferred daily for several days to restore its vigor.

7. Remove the growth from the slant with as little disturbance as possible.

8. Gently stir sufficient organisms into a tube of distilled water to produce a distinctly turbid suspension. The water should have the same temperature as that of the laboratory.

9. The organisms should not be allowed to remain in the distilled water for a longer period than 10 min.

10. Remove a loopful of the organisms from the top of the suspension and place it at one end of a glass slide, prepared as already directed.

11. Spread out the suspension thinly by touching it with the edge of a second slide and drawing it over the surface by the same method followed in preparing a blood film.

12. The film must be thin so that it will dry rapidly. This is necessary to minimize distortion.

13. Add 0.4 cc. of saturated alcoholic solution of basic fuchsin to 9.0 cc. of Gray's mordant contained in a test tube.

14. Mix by rapid rotation of the tube until a precipitate forms.

15. The mixture must be freshly prepared for each batch of slides as it deteriorates after 24 hr.

16. Filter the mixture and pour about 0.5 cc. of the filtrate on each slide to be stained.

17. Allow the stain to act for 10 min.

18. Wash off the mordant for 10 sec. in tap water.

19. Dry the slide in air without heating.

20. Cover the film with Ziehl-Neelsen's carbolfuchsin stain and allow to remain for 5 min.

21. Wash in tap water, blot, and air-dry.

22. Examine under the oil-immersion objective.

PLIMMER AND PAINE'S METHOD

1. Follow the same procedure as given for the preparation of the bacterial films.

2. Dilute Plimmer and Paine's mordant with 2 parts of distilled water in a test tube and stopper securely.

3. Invert the tube several times to mix the contents.

4. Allow to stand for about 1 min.

5. Filter the mordant onto the slide.

6. Allow to act for 1 min. A slight bronzing effect should be visible on the surface.

7. Wash the slide rapidly in tap water.

8. Cover the film with Ziehl-Neelsen's carbolfuchsin stain and allow to remain for 5 min.

9. Wash the slide in tap water, blot, and air-dry.

10. Examine under the oil-immersion objective.

Questions:

1. How is Gray's mordant prepared?

2. How is Plimmer and Paine's mordant prepared?

3. What is a mordant? How does it differ from an intensifier?

4. Why is a mordant necessary to stain flagella?

5. Why should the slides be scrupulously clean?

6. Why is a young culture better than an old one?

7. Are flagella longer than the bacterial bodies? Explain.

8. Does the number of flagella possessed by an organism determine its rate of motility?

9. Why must the inoculating loop not be used in spreading the drop on the slide?

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COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1941.

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EXERCISE 9

THE CAPSULES OF BACTERIA

Capsules are mucilaginous substances of a carbohydrate nature elaborated by the external or slime layer of the bacterial cell wall. All

bacteria are believed to produce some capsular material but a few species are surrounded by relatively large capsules, which may be readily seen when appropriately stained.

The staining of capsules is a simple technique and their presence easily recognized by microscopic examination. Capsules are composed of complex carbohydrates known as polysaccharides. Since they are water-soluble, water must be avoided as much as possible in the various staining procedures; otherwise the capsular material may be dissolved and washed away from the organisms.

Required:

1. 48-hr. milk culture of *Klebsiella pneumoniae*.
2. 1 per cent aqueous solution of crystal violet (90 per cent dye content).
3. 20 per cent aqueous solution of copper sulfate.
4. Hiss' capsule stain.
5. Clean slides.

Procedure:

ANTHONY'S METHOD

1. Prepare a smear from a 48-hr. milk culture of *K. pneumoniae*.
2. Allow the smear to air-dry; do not fix!
3. Stain the smear with a 1 per cent aqueous solution of crystal violet.
4. Allow the stain to act for 2 min.
5. Wash off the stain with a 20 per cent solution of copper sulfate, drain, blot, and air-dry.
6. Examine the slide under the oil-immersion objective.
7. The capsules appear as faint blue halos around dark-purple bodies.

HISS' METHOD

1. Prepare a smear in the same manner as given above.
2. Cover the film with Hiss' capsule stain, which consists of an aqueous solution of gentian violet.
3. Heat the slide over a low flame for $\frac{1}{2}$ min. until steam arises.
4. Wash off the stain with a 20 per cent aqueous solution of copper sulfate. Do not use water!
5. Drain the slide, blot, and air-dry.
6. Examine the slide under the oil-immersion objective.
7. As in the first method, the capsules appear as faint-blue halos around dark-purple-staining cells.

Questions:

1. Why should the use of water be avoided in staining capsules?
2. Discuss the composition of capsular material.
3. Does *K. pneumoniae* produce capsules when grown in nutrient broth? Explain.
4. What is the purpose of the copper sulfate in the staining process?
5. Are gum-producing organisms of any economic importance?

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EXERCISE 10

THE SPORES OF BACTERIA

Spores are bodies which are produced within the cells of some bacteria and which are more resistant to adverse conditions than the vegetative cells producing them. Their formation is limited almost entirely to the rod-shaped bacteria.

Sporulation is not a process to increase bacterial numbers since very few cells produce more than one spore. They are a means of keeping a species alive when conditions become too unfavorable for the existence of the vegetative cells. Spores are present in greater numbers in old rather than in young cultures.

Required:

1. 72-hr. nutrient agar slant culture of *Bacillus subtilis*.
2. Methylene blue stain.
3. 5 per cent aqueous solution of malachite green.
4. 0.5 per cent aqueous solution of safranine.
5. Clean slides.

Procedure:

SIMPLE STAINING METHOD

1. Prepare a smear from a 72-hr. nutrient agar slant culture of *B. subtilis*.
2. Dry, by holding the slide high over a low gas flame, and fix.
3. Cover the film with methylene blue stain and allow to remain for 2 min.
4. Wash the slide in tap water, drain, blot, and air-dry.
5. Examine under the oil-immersion lens.
6. The vegetative cells appear blue; the spores fail to take the stain.

SCHAEFFER AND FULTON'S METHOD

1. Prepare a smear from a 72-hr. nutrient agar slant culture of *B. subtilis*.
2. Dry, by holding the slide high over a low gas flame, and fix.
3. Cover the film with a 5 per cent aqueous solution of malachite green and allow to act in the cold for 30 to 60 sec.; then heat the slide until it steams 3 or 4 times.
4. Wash the slide in tap water for about $\frac{1}{2}$ min.
5. Cover the film with a 0.5 per cent aqueous solution of safranine and allow to stain for 30 sec.
6. Wash the slide in tap water, drain, blot, and air-dry.
7. The spores stain green; the remainder of the cells red.

Questions:

1. Why are spores more difficult to stain than vegetative cells?
2. What environmental conditions are necessary for sporulation to occur?
3. How does spore formation among the bacteria differ from sporulation in the yeasts and molds?

4. Is sporulation a process to increase bacterial numbers? Explain.

5. What reasons have been advanced to explain why spores are more resistant to adverse environmental conditions than the vegetative cells producing them?

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EXERCISE 11

THE GRAM STAIN

The Gram stain is probably the most important differential stain used by the bacteriologist.

The procedure separates bacteria into two groups depending upon whether the original stain is retained or lost when the stained smear is treated with an iodine solution and then washed in alcohol. Organisms that retain the stain when washed with alcohol are termed Gram-positive; those which fail to retain the original stain but take the counterstain are called Gram-negative.

The stain is of considerable value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient agar slant culture of *Escherichia coli*.
2. 24-hr. nutrient agar slant culture of *Bacillus subtilis*.
3. Hucker's ammonium oxalate crystal violet stain.
4. Acetone alcohol.
5. Gram's iodine solution.
6. Safranine stain.
7. Clean slides.

Procedure:

1. Prepare a smear from a 24-hr. nutrient agar slant culture of *E. coli*. in the usual manner.
2. Dry and fix.
3. Cover the film with Hucker's ammonium oxalate crystal violet stain and allow to remain for 1 min.
4. Pour off the excess stain and apply Gram's iodine solution.
5. Allow to remain for 1 min.
6. Add acetone alcohol, drop by drop, until the violet color ceases to flow away.
7. Wash the slide in tap water.
8. Counterstain with safranine for 1 min.
9. Again wash the slide in tap water, drain, blot, and air-dry.
10. Examine under the oil-immersion objective.
11. Repeat the above procedure using a 24-hr. nutrient agar slant culture of *B. subtilis*.

12. Record your results in the following table:

Organism	Color	Gram reaction
E. coli		
B. subtilis		

Questions:

1. Why is aniline oil sometimes added to gentian violet stain?
2. How is Hucker's ammonium oxalate crystal violet prepared?
3. Why is the ammonium oxalate added?
4. How do crystal violet and gentian violet differ?
5. What is the purpose of the iodine solution?
6. Why do Gram-positive organisms from an old culture sometimes stain Gram-negative?
7. Can all cells, both plant and animal, be divided into two groups on the basis of the Gram stain?
8. What factors are likely to cause variations in the Gram reaction?

References

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1939.
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EXERCISE 12

THE ACID-FAST STAIN

Some bacteria are believed to be surrounded by a covering of fatty and waxy substances and for that reason are not satisfactorily stained in the usual simple staining procedures. These organisms are not easily penetrated by stains but, when once stained, they retain the color even though treated with alcohol containing acid. Because of this fact the organisms are said to be acid-fast.

This differential staining procedure is of great value for the identification of the organisms of tuberculosis and leprosy, the two most important members of the acid-fast group.

Required:

1. Specimen of autoclaved sputum from a tuberculous patient.
2. 24-hr. nutrient broth culture of *Escherichia coli.*
3. 24-hr. nutrient broth culture of *Bacillus subtilis.*
4. Ziehl-Neelsen's carbolfuchsin stain.
5. Acid alcohol.
6. Methylene blue stain.
7. Clean slides.

Procedure:

THE COLD METHOD

1. Prepare a smear from the tuberculous sputum in the usual manner.
2. Dry and fix.
3. Cover the film with Ziehl-Neelsen's carbolfuchsin stain and allow to remain for 20 min.
4. Wash the slide in tap water.
5. Decolorize the smear with acid alcohol until the color ceases to flow away.
6. Wash the slide in tap water.
7. Cover the film with methylene blue stain and allow to act for 1 min.
8. Again wash the slide in tap water, drain, blot, and air-dry.
9. Examine under the oil-immersion objective.

THE HEAT METHOD

1. Prepare a smear from the autoclaved tuberculous sputum.
2. Dry and fix.
3. Rest the slide on a piece of wire gauze placed on a tripod.
4. Cover the film with Ziehl-Neelsen's carbolfuchsin stain.
5. Gently heat the slide until the stain just steams. Do not allow the stain to boil!
6. Continue to heat for about 3 min.
7. Add more stain as evaporation takes place to keep the smear moist during the heating period.
8. Wash the slide in tap water.
9. Decolorize the smear with acid alcohol until the color ceases to flow away
10. Wash the slide in tap water.
11. Cover the film with methylene blue stain and allow to act for 1 min.
12. Again wash the slide in tap water, drain, blot, and air-dry.
13. Examine under the oil-immersion objective.
14. Prepare a smear from a 24-hr. nutrient broth culture of *E. coli.* and another from a 24-hr. nutrient broth culture of *B. subtilis.*
15. Dry and fix.
16. Stain by either the cold or heat method.
17. Make drawings and record your observations in the following table:

Organism	Acid-fast stain		Gram stain	
	Positive	Negative	Positive	Negative
M. tuberculosis				
E. coli				

Questions:

1. How is acid alcohol prepared?
2. Could acetone alcohol be substituted for the acid alcohol?
3. Does the acid-fast stain correlate with the Gram stain?
4. Are Gram-negative organisms ever acid-fast?
5. If tubercle bacilli were treated with a fat solvent to remove the fatty and waxy material, would the cells still stain acid-fast?   Explain.

References

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1939.

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THE MORPHOLOGY OF YEASTS AND MOLDS

EXERCISE 13

THE MORPHOLOGY OF GROWING YEAST

The yeasts of industrial importance are members of the genus *Saccharomyces*. This includes *Saccharomyces cerevisiae* commonly known as baker's or brewer's yeast. It is the same species that is employed medicinally and for the fermentation of sugar to alcohol. The cells are round, oval, or elongated.

S. cerevisiae reproduces by budding and by asexual spore formation. Sexual multiplication has not been observed. Asci (spore sacs) contain from one to four round, smooth spores. The spores germinate by budding.

Required:

1. 24-hr. glucose broth culture of *Saccharomyces cerevisiae.*
2. Slides.
3. Cover slips

Procedure:

1. Place two or three loopfuls of a 24-hr. glucose broth culture of *S. cerevisiae* on a glass slide and cover lightly with a cover slip.
2. Focus with the low-power objective; then swing the high-power objective into place.
3. Examine the yeast cells carefully.
4. Note the presence of nucleuses, vacuoles, granules, cell walls, buds, etc.

Questions:

1. How do the cells multiply?
2. Does *S. cerevisiae* produce spores?
3. What name is given to yeasts that do not produce spores?
4. Do yeasts contain a well-defined nucleus?
5. What is the composition of the cell walls of yeasts?
6. When are granules especially prominent in yeast cells?
7. Are yeasts motile?
8. How are yeasts differentiated from bacteria?

References

HENRICI, A. T.: The Yeasts: Genetics, Cytology, Variation, Classification and Identification, *Bact. Rev.*, **5**: 97, 1941.

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EXERCISE 14

SPORULATING YEAST

Saccharomyces cerevisiae seldom produces spores in glucose broth. Special methods are required to cause the organisms to sporulate.

The following conditions must be satisfied:

1. There must be an abundant supply of oxygen.
2. The medium must be deficient in nutrients.
3. A low temperature (room) must be used.
4. The yeast cells must be young and vigorous.

These conditions are readily fulfilled by streaking the organisms over the surface of a plaster of Paris block partly immersed in distilled water and storing the culture in the laboratory desk.

Required:

1. A vigorously growing 24-hr. glucose agar slant culture of *Saccharomyces cerevisiae*. This culture is prepared by making daily transfers over a period of about 3 days.
2. Sterile Petri dish containing a block of plaster of Paris.
3. Tube of sterile distilled water.
4. 5 per cent aqueous solution of malachite green.
5. 0.5 per cent aqueous solution of safranine.
6. Clean slides.

Procedure:

1. Pour the sterile distilled water into the Petri dish containing the plaster of Paris block. Observe aseptic precautions.
2. Remove two or three loopfuls of the yeast growth from the surface of the glucose agar slant culture and smear over the surface of the plaster of Paris block.
3. Incubate the Petri dish in your locker (room temperature) for 48 hr.
4. Make a smear from the material on the surface of the block.
5. Dry over a flame and fix.
6. Stain by Schaeffer and Fulton's method (see page 24).
7. Examine under the oil-immersion objective.

Questions:

1. What is an ascospore?
2. How many spores are usually found in each cell of baker's yeast?
3. How does this compare with the number found in a bacterial cell?
4. Are ascospores motile?

References

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Exercise 15

THE MORPHOLOGY OF SOME COMMON MOLDS

The molds commonly encountered are members of the genera *Mucor, Rhizopus, Trichothecium, Oospora, Monilia, Aspergillus, Penicillium, Cladosporium,* and *Alternaria.*

Mold specimens are very difficult to remove from culture media without being greatly broken. Therefore, great care must be exercised in preparing satisfactory mounts. Water should not be used for the mounting fluid since it rapidly evaporates, produces a shrinkage of the hyphae by osmosis, and causes the various parts to adhere together as a tangled mass. Such preparations are unsatisfactory for accurate observations.

Probably the most useful mounting medium is known as lactophenol. This fluid does not cause shrinkage of the cells and does not evaporate, thus permitting permanent preparations to be prepared. A dye may be added to the fluid to stain the various mold structures.

Required:

1. Molds from various materials such as fruits, vegetables, bread, or contaminated culture media.
2. Clean slides.
3. Clean cover slips.
4. Solution of lactophenol.

Procedure:

1. Place two or three loopfuls of a solution of lactophenol in the center of a clean slide.
2. Remove a small portion of the mold from the contaminated material and place in the lactophenol solution on the slide.
3. Gently tease the material with a pair of needles until the various structures are well separated and wetted by the fluid.
4. Cover with a clean cover slip.
5. Focus with the low-power objective; then swing the high-power objective into place.
6. Note the following and sketch in your notebook: (a) form of the mycelium, (b) branched or unbranched, (c) septate or nonseptate, (d) internal structure of mycelium, (e) sterigma, (f) sporangium, (g) conidiophore, (h) conidia, (i) shape of conidia, and (j) zygospore.

Questions:

1. How do molds differ from yeasts? From bacteria?
2. What is chitin and where is it found?
3. Are mold spores more resistant to unfavorable conditions than bacterial spores?
4. Do all molds produce both sexual and asexual spores?
5. Why are culture media frequently contaminated by molds?

References

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Smith, G.: "An Introduction to Industrial Mycology," London, Edward Arnold & Co., 1938.

THE NUTRITION OF BACTERIA

EXERCISE 16

THE PREPARATION OF NUTRIENT BROTH

Nutrient broth is probably the most commonly employed bacteriological medium. It is a simple medium, being composed of peptone, beef extract, and distilled water. Sometimes sodium chloride is added.

Required:

1. Beef extract.
2. Peptone.
3. Distilled water.
4. Pan.

Procedure:

1. Mix 3 gm. of beef extract and 5 gm. of peptone with 1000 cc. of distilled water (med. 29, page 181).
2. Bring the solution to a boil.
3. Make up the loss due to evaporation.
4. Pour the medium into a flask and preserve for Exercises 17, 18, and 19.

Questions:

1. Why is beef extract added to the medium?
2. What is peptone and why is it used?
3. Could tap water be used instead of distilled water? Explain.
4. What is the difference between nutrient broth and infusion broth?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 17

THE ADJUSTMENT OF REACTION OF NUTRIENT BROTH TO pH7.0

Culture media are adjusted to different hydrogen-ion concentrations, depending upon the growth requirements of the organisms to be cultivated. Some organisms grow best in acid media; others require alkaline environments; still others prefer media that are neither acid nor alkaline but neutral in reaction.

The method generally followed for adjusting the reaction of media is to determine the actual numbers of free hydrogen ions present. This can be carried out either colorimetrically or electrometrically. For con-

venience the colorimetric method is generally followed although it is not so accurate as the electrometric method.

Required:

1. 500 cc. nutrient broth (med. 29, page 181).
2. Bromothymol blue indicator solution.
3. 1N NaOH solution.
4. 0.05N NaOH solution.
5. Comparator block.

Top View

Front View
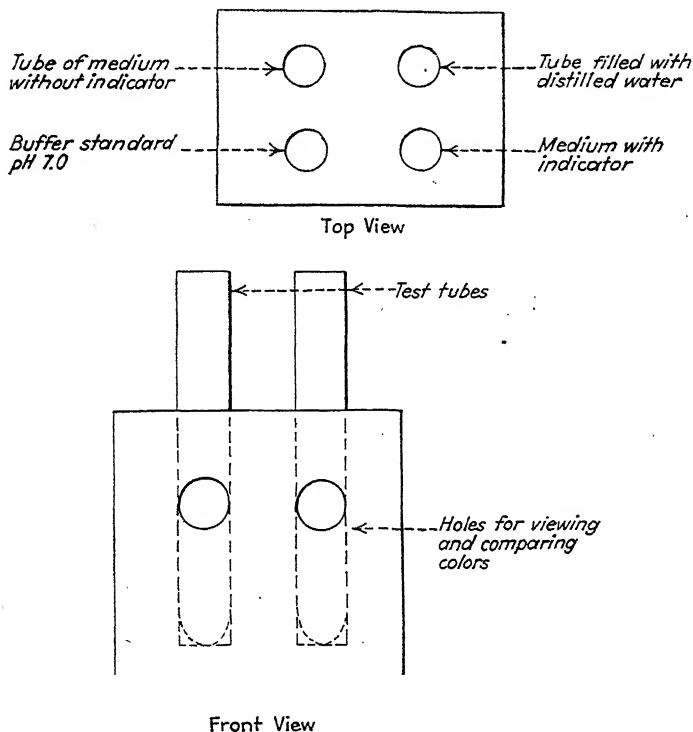
FIG. 2.—Comparator block.

6. Test tubes for comparator block.
7. Burettes.
8. Pipettes.
9. Plugged test tubes.
10. Funnel.
11. Filter paper.

Procedure:

1. Pipette 5 cc. of the medium into each of two test tubes.
2. Add 0.5 cc. of bromothymol blue indicator to only one of the tubes. Mix thoroughly.
3. If the medium is acid, the color will be yellow; if alkaline, it will be blue. neutral reaction (pH7.0) is indicated by a grass-green color.

4. Set up a comparator block as shown in Fig. 2.

5. The purpose of the tube of medium behind the standard is to compensate for the color of the medium.

6. The tube of distilled water behind the tube of medium with indicator compensates for the depth of liquid when viewing the color through the two holes.

7. Hold the block up to the level of the eyes and face the light when making readings.

8. If the medium with indicator is yellow, add $0.05N$ NaOH, drop by drop, until the color matches that of the standard.

9. If the color is blue, add $0.05N$ HCl until the color matches that of the standard. Read the burette.

10. From the number of cubic centimeters of $0.05N$ NaOH or $0.05N$ HCl required to adjust 5 cc. of the medium, calculate the number of cubic centimeters needed to adjust the total amount (490 cc.).

11. Since the amount of $0.05N$ alkali or acid required may cause too great dilution of the medium, adjust the balance of the broth (490 cc.) with $1N$ alkali or acid.

The following example will show the method of calculation:

5 cc. of medium titrated.

0.3 cc. $0.05N$ NaOH required.

490 cc. = total amount of medium.

$$\frac{5}{490} :: \frac{0.3}{X} \qquad 5X = 147.0$$

$$X = 29.4 \text{ cc., } 0.05N \text{ NaOH}$$

$\frac{29.4}{20} = 1.47$ cc. $1N$ NaOH required to adjust 490 cc. of nutrient broth to pH7.0.

12. Check the medium after the addition of the $1N$ alkali to make sure your calculations are correct. If not, readjust again.

13. Filter the medium through paper.

14. Fill 10 test tubes with approximately 7 cc. of medium in each.

15. Label with your name and desk number.

16. Autoclave at 15-lb. pressure for 30 min.

17. Store the tubes in your locker.

18. Use the remainder of the broth for the preparation of nutrient agar and nutrient gelatin.

Questions:

1. Is beef extract acid or alkaline in reaction? Why?

2. Why is the medium filtered after the adjustment of the reaction rather than before?

3. Does the reaction of the medium change during sterilization?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 18

THE PREPARATION OF NUTRIENT AGAR

Required:

1. 100 cc. of nutrient broth (Exercise 17).

2. Agar.

   3. Pan.
   4. Bromothymol blue indicator solution.
   5. Comparator block.
   6. Test tubes for comparator block.
   7. 1*N* NaOH solution.
   8. 0.05*N* NaOH solution.
   9. Burettes.
  10. Pipettes.
  11. Cotton.
  12. Funnel.
  13. Plugged test tubes.

Procedure:

   1. Pour 100 cc. of nutrient broth into a pan or bowl and add 2 gm. of agar.
   2. Boil the medium until the agar is dissolved.
   3. Make up loss due to evaporation with distilled water.
   4. The addition of the agar may or may not change the original reaction, depending upon its purity. Therefore, check the reaction of the finished medium following the same procedure as given under Exercise 17. If it has become more acid, readjust the reaction to pH7.0.
   5. Filter the medium through cotton.
   6. Fill five test tubes with approximately 10 cc. and five tubes with approximately 5 cc. of medium in each.
   7. Label with your name and desk number.
   8. Autoclave at 15-lb. pressure for 30 min.
   9. Place the tubes containing 5 cc. of agar in an inclined position so that a surface is formed with very little butt. Do not allow the upper portion of the slant to touch the cotton stopper.
  10. When the agar has solidified, store both lots of tubes in your desk.

Questions:

   1. What is agar?
   2. Does it have other uses commercially?
   3. Why do some lots of agar, after being added to clear broth, cause a precipitate to form?
   4. Why is agar preferred to gelatin as a solidifying agent?
   5. At what temperature does agar dissolve? Solidify?

<div align="center">Reference</div>

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw Hill Book Company, Inc., 1942.

<div align="center">EXERCISE 19</div>

<div align="center">THE PREPARATION OF NUTRIENT GELATIN</div>

Required:

   1. 100 cc. of nutrient broth (Exercise 17).
   2. Gelatin.
   3. Pan.
   4. Bromothymol blue indicator solution.

5. Comparator block.
6. Test tubes for comparator block.
7. 1N NaOH solution.
8. 0.05N NaOH solution.
9. Burettes.
10. Pipettes.
11. Cotton.
12. Funnel.
13. Plugged test tubes.

Procedure:

1. Pour the broth into a pan or bowl and add 15 per cent gelatin.
2. Heat the medium until the gelatin is dissolved.
3. Make up the loss due to evaporation with distilled water.
4. The addition of gelatin usually causes the reaction of the broth to become more acid. Therefore, check the reaction of the finished medium following the same procedure as given under Exercise 17. If it has become more acid readjust the reaction to pH7.0.
5. Filter the medium through cotton.
6. Fill 10 test tubes with approximately 7 cc. of medium in each.
7. Label with your name and desk number.
8. Sterilize in an Arnold for 20 min. at 100°C. on three successive laboratory periods.
9. Store the tubes in your desk.

Questions:

1. What is gelatin?
2. Is it added to culture media for its food value?
3. Why is it not used in place of agar as a solidifying agent?
4. Why is gelatin sterilized in an Arnold sterilizer?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 20

THE PREPARATION OF BROMOCRESOL PURPLE MILK

Required:

1. Fresh milk.
2. 1.6 per cent solution of bromocresol purple.
3. Pipettes.
4. Plugged test tubes.

Procedure:

1. Remove the cream layer from fresh milk. This is best carried out by using a siphon.
2. To each liter of skimmed milk add 1 cc. of a 1.6 per cent solution of bromocresol purple and mix well.

3. Pipette approximately 7 cc. of medium into each of 10 test tubes.

4. Sterilize in an Arnold for 20 min. at 100°C. on three successive laboratory periods.

5. Label with your name and desk number.

6. Store in your desk.

Questions:

1. Why is bromocresol purple used in preference to other indicators?

2. Why is the cream layer removed?

3. What is the chief use of this medium in bacteriology?

4. Why is the medium sterilized in an Arnold?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 21

BUFFERS

Substances that possess the power of resisting changes in acidity or alkalinity are spoken of as buffers. The commonly used buffers are salts of weak acids. These include phosphates, carbonates, citrates, etc. In addition, amphoteric compounds such as proteins and protein split products (proteoses, peptones, peptids, amino acids) are also efficient buffers.

Required:

1. 2 tubes of nutrient broth each containing 5 cc.

2. 2 tubes of distilled water each containing 5 cc.

3. 2 tubes of 1 per cent primary sodium phosphate solution each containing 5 cc.

4. 2 tubes of 1 per cent secondary sodium phosphate solution each containing 5 cc.

5. Bromocresol green indicator solution.

6. 0.1N HCl solution.

7. Pipettes.

Procedure:

1. Add 5 drops of bromocresol green indicator solution to each of the two tubes of distilled water. Mix and note the color.

2. Add 1 drop of 0.1N HCl to one of the tubes. Shake and record the color.

3. Add 5 drops of bromocresol green indicator to each of the two tubes of nutrient broth. Shake and note the color.

4. To one of the tubes, add 0.1N HCl drop by drop, until approximately the same tint as under (2) is obtained. Record the number of drops.

5. Add 5 drops of bromocresol green indicator solution to each of the two tubes containing 1 per cent Na_2HPO_4 solution. Shake and note the color.

6. To one of the tubes add 0.1N HCl drop by drop, until approximately the same tint as under (2) is obtained. Record the number of drops.

7. Repeat the experiment using 1 per cent NaH_2PO_4 solution.

8. Record all results in the following table:

Medium	Color after addition of bromocresol green indicator	Approximate pH	Number of drops 0.1N HCl added	Approximate pH
Distilled water				
Nutrient broth				
Na$_2$HPO$_4$ solution				
NaH$_2$PO$_4$ solution				

9. Interpret your results.

Questions:

1. Name some of the buffers commonly added to culture media.
2. Many buffers are known but only a few are incorporated in culture media. Why?
3. What buffer is commonly present in culture media?
4. Is sodium chloride a buffer?
5. Which is the better buffer, NaH$_2$PO$_4$ or Na$_2$HPO$_4$?   Explain.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Various methods are employed for the inoculation of different kinds of culture media. Any method may be followed provided it produces satisfactory results and may be performed in a minimum of time. The following procedures are recommended.

Agar Deep Cultures.—Sterilize the wire needle in a flame and allow it to cool for a few moments. Remove the cotton stopper from an agar slant culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contamination. Remove a small amount of the growth with the sterilized wire needle. Again flame the neck of the agar slant culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the tube to be inoculated by grasping it with the small finger of the right hand. Flame the neck of the tube. Stab the straight wire containing the inoculum to the bottom of the tube. Withdraw the needle carefully. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle before setting it down on the table. Mark the tube with the name of the organism and the date. Use a china marking pencil. Incubate the culture at the proper temperature.

If a transfer is to be made from a liquid culture, use the wire loop instead of the needle. Remove a loopful of the culture and force the wire to the bottom of the tube of agar. Withdraw the loop carefully. The procedure in every other detail is the same as the foregoing.

Agar Slant Cultures.—Sterilize the wire needle or wire loop in the flame, depending upon whether a solid or a liquid culture is to be used. Allow the wire to cool for a few moments. Remove the cotton stopper from the culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contamination. Remove a small amount of the growth with the sterilized wire needle, or a loopful of the liquid culture with the wire loop. Again flame the neck of the culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the agar slant to be inoculated by grasping it with the small finger of the right hand. Flame the neck of the tube. Spread the inoculum over the surface of the agar slant by making streaks back and forth a few millimeters apart. Start at the butt of the slant and work up to the top. Withdraw the needle or loop from the tube. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle or loop before setting it down on the table. Mark the tube

with the name of the organism and the date.   Use a china marking pencil.
Incubate the culture in an upright position at the proper temperature.

Broth Cultures.—The same procedure is followed as given under Agar
Slant Cultures except that the inoculum is transferred to broth.   If
growth from an agar slant culture is transferred to broth, vigorous shaking
may be necessary to dislodge the inoculum from the wire.   If a liquid
inoculum is used, only gentle shaking is necessary to remove the loop
of culture from the wire before withdrawing from the tube.

<div align="center">

EXERCISE 22

THE ISOLATION OF PURE CULTURES BY PLATING METHODS

</div>

The exercise demonstrates how a mixture of two organisms may be
separated and pure cultures of each obtained.

Two general methods are followed: (1) the streak-plate method and
(2) the pour-plate method.

Required:
>   1. 6 tubes of nutrient agar.
>   2. 6 sterile Petri dishes.
>   3. Culture containing a mixture of *Escherichia coli* and *Staphylococcus aureus*.
>   4. Thermometer.
>   5. 4 nutrient agar slants.

Procedure:

<div align="center">

ISOLATION OF SPECIES BY THE STREAK-PLATE METHOD

</div>

1. Melt three tubes of nutrient agar in a pan of boiling water or in an Arnold
sterilizer.

2. Cool the agar to a temperature of 50°C.   Use a thermometer.

3. The cooling may be hastened by setting the tubes in a pan of lukewarm water.

4. Pour the cooled agar into three sterile Petri dishes and set aside until firm.

5. Remove a loopful of the mixed culture from the tube.

6. Spread the loopful of the culture at the upper end of the Petri dish to thin it
out.   Then make streaks back and forth over the surface of the agar about ¼ in.
apart.

7. Each streak will leave a bit of the bacterial mixture on the agar.

8. The first streaks will contain too many organisms with the result that well-
isolated colonies will not be obtained.   The last streaks should thin out the organisms
sufficiently to give well-separated colonies.

9. It is better to streak a second plate without reinoculating the wire loop.
This gives greater certainty in securing well-isolated colonies.

10. The third plate is not inoculated but serves as a control for both the streak-
plate and the pour-plate methods.   It acts as a check on the sterility of the agar and
Petri dishes and, also, on the technique of preparing the plates.

11. Invert the plates and incubate them at 37°C. for 48 hr.

12. The plates are inverted to prevent the accumulation of water of condensation
on the surface of the agar.   If not inverted, the moisture will cause the growth to
spread and prevent the development of well-isolated colonies.

13. After incubation well-isolated colonies should appear on one of the streaked
plates.

14. Each separate colony usually represents the growth from a single organism.

15. The control plate should remain sterile.

16. Examine the plates for the presence of two different kinds of colonies.

17. Select one well-isolated colony of each species.

18. Remove a portion of one of the colonies with the wire needle and emulsify the growth in a loopful of distilled water on a slide.

19. Do likewise with the other colony.

20. Dry the smears, fix, and stain by the Gram technique.

21. Examine the slides under the oil-immersion objective.

22. On one slide you should have a pure culture of a Gram-positive coccus and on the other a pure culture of a Gram-negative rod. If you do not have a pure colony of each organism, continue to examine well-isolated colonies until you have succeeded.

23. Transfer the remaining portions of the two different colonies to nutrient agar slants.

24. Incubate the tubes at 37°C. for 24 to 48 hr.

25. Again examine the cultures for purity by preparing Gram stains.

26. If you do not have more than one species of organism in each tube, you have succeeded in isolating pure cultures from a bacterial mixture.

ISOLATION OF SPECIES BY THE POUR-PLATE METHOD

1. A culture containing two or more bacterial species must be highly diluted before it can be used. Unless this is practiced, the colonies will develop so close together that isolations will be practically impossible.

2. Melt three tubes of nutrient agar in a pan of boiling water or in an Arnold sterilizer.

3. Cool the agar to a temperature of 50°C. Use a thermometer.

4. The cooling may be hastened by setting the tubes in a pan of lukewarm water.

5. When the agar has cooled to the required temperature, inoculate one tube with a loopful of the bacterial mixture.

6. Mix thoroughly by rotating the tube between the palms of the hands.

7. Remove one loopful of the inoculated and mixed agar and transfer to a second tube.

8. Again rotate the tube between the palms of the hands to mix thoroughly.

9. Remove one loopful from the second inoculated tube and transfer to the third tube.

10. Mix thoroughly as before.

11. Pour the inoculated agar into three sterile Petri dishes. Observe aseptic precautions throughout.

12. Unless you work fast there is danger of the agar solidifying, with the result that the experiment will have to be repeated.

13. Label the plates, invert, and incubate at 37°C. for 24 to 48 hr.

14. By making dilutions of the original culture in agar and pouring into Petri dishes it is possible to obtain a plate showing well-isolated colonies.

15. The first agar dilution usually contains too many organisms. This shows the presence of minute colonies very close together. The colonies are so crowded that they are not able to develop to their normal size. The second and third plates generally show colonies well isolated and of normal size.

16. From this point follow the same procedure as given under Streak-plate Method, Par. 16.

Questions:

1. Why should melted agar be cooled to about 50°C. before being poured into plates?

2. Why should melted agar be cooled to about 50°C. before being inoculated?

3. Why should inoculations for the pour-plate method be made as quickly as possible?

4. Why are colonies developing on a heavily seeded plate smaller than those appearing on a sparsely seeded plate?

5. Why are colonies growing on the surface of agar larger than those embedded in the agar?

6. Which one of the two methods is considered the better?

7. Which method is more convenient to perform?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 23

THE PRESERVATION OF LABORATORY CULTURES

Bacteria for class use are generally preserved on nutrient agar slants. There are some exceptions, such as certain pathogenic organisms, which require the addition of serum, blood, and other special constituents to the medium; anaerobic organisms, which require the presence of tissue to remove the dissolved oxygen; certain soil organisms, which require special inorganic media; etc. Such preparations are known as stock cultures.

The toxic metabolic waste products secreted by organisms diffuse into the porous agar and away from the bacteria. The bacteria are able to survive longer than when grown in a liquid medium where they are bathed by the toxic substances dissolved in the surrounding nutrient solution.

This does not mean that bacteria can survive indefinitely on a solid medium. Owing to dehydration of the medium, to saturation of the agar with metabolic products, to exhaustion of the nutrients, etc., the organisms will die unless transferred to fresh medium at frequent intervals.

Stock cultures should be transferred to fresh media about once a month, incubated at 37°C. for 24 hr., then stored away in a cool dark room or cupboard. Transfers for class use are prepared from the stock cultures.

Required:

1. Nutrient broth culture of *Escherichia coli*.

2. Nutrient broth culture of *Bacillus subtilis*.

3. 2 nutrient agar slants

Procedure:

1. Inoculate a nutrient agar slant with one loopful of a broth culture of *E. coli*.

2. Inoculate a second nutrient agar slant with one loopful of a broth culture of *B. subtilis*.

3. Write the name of the organism and date on each tube with a china marking pencil.

4. Incubate the tubes at 37°C. for 24 hr.

5. Remove the cultures from the incubator and store in your locker for future use.

Questions:

1. Why are solid media more satisfactory than liquid media for the preservation of cultures?

2. Why should stock cultures be preserved in a cool place?

3. Would a humid atmosphere be better than a dry one for storing stock cultures? Explain.

4. Which method is generally considered to be the better for the preservation of organisms for indefinite periods?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 24

THE DETERMINATION OF BACTERIAL SPECIES

Each student will be required to identify a bacterial unknown. The descriptive chart found at the end of this exercise may be used. If additional charts are required they may be purchased from the Society of American Bacteriologists, Geneva, N. Y.

The unknowns will be issued by the instructor in charge. They may be made up of one or more than one organism. The bacteria used in preparing the unknowns will be those you have worked with in the various exercises of this Manual. After the blank spaces on the chart have been filled, an attempt should be made to name the bacterial species.

The books named below will be found helpful in classifying the organism or organisms studied. The Introduction to "Bergey's Manual" gives an excellent outline of suggestions for identifying unknown organisms.

Questions:

1. What should be the first step in attempting to identify an unknown organism?

2. Does the examination of a smear preparation from the original unknown tell you how many bacterial species are present?

3. Why is it a difficult matter to classify an unknown organism?

4. Do changes in various environmental factors produce changes in bacterial characteristics?

5. Can bacteria be made to acquire new characteristics?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Name of organism _____ Source _____
Date of isolation _____ Habitat _____
Is phase variation observed? _____ Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) _____

Underscore required terms.	SKETCHES	

VEGETATIVE CELLS: Medium used _____
  Reaction (pH) _____ Temp. _____ Age ____ d.
  Size of Majority _____
  Ends, *rounded, truncate, concave, tapering*
MOTILITY: In broth _____ On agar _____
SPORANGIA and ENDOSPORES: *present, absent.*
  Medium used ____ pH ____ Temp. ____ Age ___ d.
  Endospore Form: *spherical, ellipsoid, cylindrical*
IRREGULAR FORMS:
  Present on _____ in _____ days at _____ °C.

AGAR COLONIES: Temperature _____ °C. Age ____ d.
  Form, *punctiform (i.e. under 1 m.m. diam.), circular (i.e. over 1 m.m. diam.), filamentous, irregular, rhizoid.*
  Surface, *smooth, rough, concentrically ringed, radiately ridged.*
  Edge, *entire, undulate, lobate, erose, filamentous, curled.*
  Elevation of growth, *effuse, flat, raised, convex.*
  Optical Characters, *opaque, translucent, opalescent, iridescent.*

Surface Colonies   *Deep Colonies*

GELATIN COLONIES: Temperature _____ °C. Age ____ d.
  Form, *punctiform, circular, irregular, filamentous.*
  Elevation, *flat, raised, convex, pulvinate, crateriform (liquefying).*
  Edge, *entire, undulate, lobate, erose, filamentous, curled.*
  Liquefaction, *cup, saucer, spreading.*
  Surface, *smooth, contoured, rugose.*
  Optical Characters, *opaque, translucent, opalescent, iridescent.*

Surface Colonies   *Deep Colonies*

AGAR STROKE: Temperature _____ °C. Age ____ d.
  Growth, *scanty, moderate, abundant, none.*
  Form of growth, *filiform, echinulate, beaded, spreading, arborescent, rhizoid.*
  Lustre, *glistening, dull.*
  Chromogenesis _____ *photogenic, fluorescent.*
  Odor, *absent, decided, resembling* _____
  Consistency, *butyrous, viscid, membranous, brittle.*
  Medium, *grayed, browned, reddened, blued, greened, unchanged.*

Medium:
Temperature
_____ °C.
Age
_____ d.

NUTRIENT BROTH: Temperature _____ °C. Age ____ d.
  Surface growth, *ring, pellicle, flocculent, membranous, none.*
  Clouding, *slight, moderate, strong, transient, persistent, none, fluid turbid, granular growth.*
  Odor, *absent, decided, resembling* _____
  Sediment, *compact, flocculent, granular, flaky, viscid.*
  Amount of sediment, *abundant, scanty, none.*

Medium:
Temperature
_____ °C.
Age
_____ d.

GELATIN STAB: Temperature _____ °C. Age ____ d.
  Growth, *uniform, best at top, best at bottom.*
  Line of puncture, *filiform, beaded, papillate, villous, arborescent.*
  Liquefaction, *none, crateriform, infundibuliform, napiform, saccate, stratiform:* begins in ____ d. complete in ____ d.
  Degree of liquefaction in _____ days _____
  Method used _____
  Medium, *fluorescent, browned, unchanged.*

Medium:
Temperature
_____ °C.
Age
_____ d.

Fermentation   Temperature _____ °C

	Monosaccharides							Disaccharides						Polysaccharides						Alcohols							Glucosides			
Medium containing _____ _____ and:	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Melibiose	Cellobiose	Raffinose	Melizitose	Starch	Inulin	Dextrin	Glycogen	Glycerol	Erythritol	Arabitol	Adonitol	Mannitol	Sorbitol	Dulcitol	Salicin	Aesculin	Coniferin	a-Methyl Gluc.
Gas in fermentation tube																														
Amt. CO_2 in Eldredge tube																														
Reaction (pH) after ____ d.																														
Titrable acidity in ml. of N/ ____ Na OH																														

Studied by _____ Culture No. _____
Optimum conditions: Media _____ Temp. _____ °C.
Phases recorded on other charts: _____

Brief Characterization

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below. In case any of these characteristics are doubtful or have not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined; V, variable; X, doubtful.

Morphological	**Vegetative Cells**	Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinae; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous	
		Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ	
		Gram stain: 0, negative; 1, positive	
		Flagella: 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined	
		Capsules: 0, absent; 1, present	
		Chains (4 or more cells): 0, absent; 1, present	
	Sporangia: 0, absent; 1, elliptical; 2, short rods; 3, spindled; 4, clavate; 5, drumsticks		
	Endospores: 0, absent; 1, central to excentric; 2, subterminal; 3, terminal		
Cultural	**Agar Stroke**	Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty	
		Lustre: 1, glistening; 2, dull	
	Agar Colonies	Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular	
		Surface: 1, smooth; 2, contoured; 3, rugose	
	Gelatin Colonies	Form: 1, punctiform; 2, circular (over 1 mm.); 3, irregular; 4, filamentous	
		Surface: 1, smooth; 2, contoured; 3, rugose	
Physiological	Biologic relationships: 1, pathogenic for man; 2, for animals but not for man; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic		
	Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe; 4, microaerophile		
	In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite		
	Chromogenesis: 0, none; 1, pink; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, black		
	Other photic characters: 0, none; 1, photogenic; 2, fluorescent; 3, iridescent		
	Indole: 0, negative; 1, positive		
	Hydrogen sulfide: 0, negative; 1, positive		
	Hemolysis: 0, negative; 1, positive		
	Methemoglobin: 0, negative; 1, positive		
	Protein Liquefaction or Digestion	Gelatin: 0, negative; 1, positive	
		Casein: 0, negative; 1, positive	
		Egg albumin: 0, negative; 1, positive	
		Blood serum: 0, negative; 1, positive	
	Indicator Reduction	Litmus: 0, negative; 1, positive	
		Methylene blue: 0, negative; 1, positive	
		Janus green: 0, negative; 1, positive	
	Rennet production: 0, negative; 1, positive		

Temperature Relations

Medium _____ pH _____
Optimum temperature for growth _____ °C.
Maximum temperature for growth _____ °C.
Minimum temperature for growth _____ °C.

THERMAL DEATH POINT: Time 10 minutes: _____ °C.

    Medium _____ pH _____

THERMAL DEATH TIME:

    Medium _____ pH _____

Temp.	Time	Temp.	Time
___°C.	___ min.	___°C.	___ min.
___°C.	___ min.	___°C.	___ min.
___°C.	___ min.	___°C.	___ min.
___°C.	___ min.	___°C.	___ min.
___°C.	___ min.	___°C.	___ min.

Chromogenesis

Gelatin _____
Agar_____
Potato _____

Other Photic Characters

Photogenesis on _____
Iridescence on _____
Fluorescence in _____

Relation to Reaction (pH) of Medium

Medium _____
Optimum for growth: *about pH*_____
Limits for growth: *from pH* _____ *to* _____

Relation to Free Oxygen

Method _____
Medium _____ Temp. _____ °C.
Aerobic growth: *absent, present, better than anaerobic growth, micro-aerophilic*
Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*
Additional data: _____

Milk

Temperature _____ °C.

Reaction:	___ d. ___;	___ d. ___;	___ d. ___
Acid curd:	___ d. ___;	___ d. ___;	___ d. ___
Rennet curd:	___ d. ___;	___ d. ___;	___ d. ___
Peptonization:	___ d. ___;	___ d. ___;	___ d. ___

Litmus Milk

Temperature _____ °C.

Reaction:	___ d. ___;	___ d. ___;	___ d. ___
Acid curd:	___ d. ___;	___ d. ___;	___ d. ___
Rennet curd:	___ d. ___;	___ d. ___;	___ d. ___
Peptonization:	___ d. ___;	___ d. ___;	___ d. ___

Reduction of litmus *begins in* _____ days, *ends in* _____ days

PATHOLOGY

Animal Inoculation

Medium used _____ Age of culture _____ Amount _____ Incubation period _____

		Whole culture		Cells		Filtrate	
Animal							
Type of Injection	Subcutaneous	*					
	Intraperitoneal						
	Intravenous						
	Per os						

* In each instance where pathogenicity is observed, indicate location of lesion, and type, *e.g.*, edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

Antigenic Action

Animal _____ Medium used _____ Age of culture _____
Type injection _____ Number of injections _____
Culture causes production of *cytolysins, agglutinins, precipitins, antitoxin.*
Specificity: Antibodies produced effective against other antigens as follows _____

Immune sera from _____
_____ effective against this organism as antigen

TARY DATA

Action on Erythrocytes	Reduction of Indicators

Action on Erythrocytes

Cells: _____
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

Production of Indole

Medium _____
Test used _____
Indole *absent, present in* _____ *days*

Production of Hydrogen Sulfide

Medium _____
Test used _____
H₂S *absent, present in* _____ *days*

Action on Nitrates

Medium _____ Temp. _____ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas (N₂): ___d.___; ___d.___; ___d.___; ___d.___
Medium _____ Temp. _____ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas (N₂): ___d.___; ___d.___; ___d.___; ___d.___
Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in _____ medium: *negative, positive*
Disappearance of 2 p.p.m. nitrite in _____ medium: *negative, positive*

Reduction of Indicators

Medium _____ pH _____ Temp. _____ °C.
Indicator   Conc.   Reduction:
_____ _____ % ___ hr. ___; ___ hr. ___
_____ _____ % ___ hr. ___; ___ hr. ___
_____ _____ % ___ hr. ___; ___ hr. ___
_____ _____ % ___ hr. ___; ___ hr. ___

Staining Reactions

Gram: ___ d. ___; ___ d. ___; ___ d. ___; ___ d. ___
    Method _____
Spores: Method _____
Capsules: Method _____
    Medium _____
Flagella: Method _____
Special Stains _____

Additional Tests

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds: _____
_____
_____

SPECIAL TESTS

THE EFFECTS OF ENVIRONMENT UPON BACTERIA

Exercise 25

THE EFFECT OF TEMPERATURE

There exists for every organism an optimum temperature at which it grows best; a minimum temperature below which it fails to grow; and a maximum temperature above which growth does not occur. The growth temperature range is the number of degrees between the minimum and maximum temperatures.

The purpose of this exercise is to determine the approximate minimum, optimum, and maximum temperatures of several organisms.

Required:
1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 24-hr. nutrient broth culture of *Bacillus subtilis*.
3. 24-hr. nutrient broth culture of *Bacillus viridulus*.
4. 12 nutrient agar slants.

Procedure:
1. Inoculate four nutrient agar slants each with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
2. Inoculate four nutrient agar slants each with one loopful of a 24-hr. nutrient broth culture of *B. subtilis*.
3. Inoculate four nutrient agar slants each with one loopful of a 24-hr. nutrient broth culture of *B. viridulus*.
4. Divide the tubes into four sets and incubate at the temperatures given in the table.
5. Note the extent of growth in each case.
6. Record your observations in the following table:

Organism	Refrigerator, °C.	Room temperature, °C.	37°C.	55°C.
E. coli				
B. subtilis				
B. viridulus				

7. Use the following system for indicating the extent of growth:

$$0 = \text{no growth.}$$
$$+ = \text{poor growth.}$$
$$++ = \text{good growth.}$$
$$+++ = \text{excellent growth.}$$

Questions:

1. How can the maximum temperature of an organism be increased?

2. Name some of the environmental factors responsible for variations in the maximum, optimum, and minimum temperatures.

3. Why do organisms fail to multiply when kept at a temperature below the minimum or above the maximum?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 26

THE THERMAL DEATH POINT

The thermal death point indicates the temperature required to kill an organism after an exposure period of 10 min. under certain specified conditions. Since all organisms in a culture are not killed in the same period of time, the term thermal death rate is more appropriate.

This exercise demonstrates the method followed for the determination of the thermal death rate.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli.*
2. 24-hr. nutrient broth culture of *Staphylococcus aureus.*
3. 24-hr. nutrient broth culture of *Streptococcus lactis.*
4. 72-hr. nutrient broth culture of *Bacillus subtilis.*
5. 72-hr. nutrient broth culture of *Bacillus megatherium.*
6. Slides.
7. Water bath.
8. 6 sterile test tubes.
9. Thermometer.
10. Sterile 1-cc. pipettes.
11. 7 sterile Petri dishes.
12. 7 tubes of nutrient agar.

Procedure:

1. Each student will determine the thermal death rate of only one organism. This will be assigned by the instructor.

2. Examine the culture for purity by making a Gram stain.

3. Roll the culture between the palms of the hands to obtain a uniform suspension of the organisms.

4. Arrange seven Petri dishes on the desk.

5. With the china marking pencil record on each plate one of the temperatures given in the table opposite the organism that has been assigned to you.

6. Place 1.0 cc. of the culture in each of six sterile test tubes.

7. Place another 1.0-cc. portion in the control plate.

8. Heat the water bath to the lowest temperature given in the table.

9. Place one of the tubes with culture in the bath and allow to remain for 11 min. This includes 1 min. for the lag period.

10. At the end of this period remove the tube from the bath and pour the contents into the Petri dish. Observe aseptic precautions.

11. Raise the temperature of the bath 5°C. and repeat the operation.

12. Do likewise for the remaining temperature intervals.

13. Melt seven tubes of nutrient agar and allow to cool to 50°C.

14. Pour the melted and cooled agar into the Petri dishes and mix thoroughly by tilting the plates from side to side.

15. When the agar has hardened, invert the plates and incubate at 37°C. for 48 hr.

16. Count the colonies if possible and record the results in the following table:

Organism	Number of colonies developing at					
	55°C.	60°C.	65°C.	70°C.	75°C.	80°C.
E. coli						
Staphylococcus aureus						
Streptococcus lactis						

Organism	Number of colonies developing at					
	75°C.	80°C.	85°C.	90°C.	95°C.	100°C.
B. subtilis						
B. megatherium						

Questions:

1. Why does *B. subtilis* have a higher thermal death rate than *E. coli?*

2. Why should cultures of *B. subtilis* and *B. megatherium* be incubated for 72 hr. instead of 24 hr. before being used?

3. What is meant by the lag period?

4. Does a knowledge of the thermal death rate of an organism have any practical application?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 27

THE EFFECT OF ULTRAVIOLET LIGHT

Light rays in the ultraviolet portion of the spectrum are toxic to bacteria. The most toxic rays appear to be in the region of 2537 Å. The light rays not only are toxic to microorganisms but produce certain harmful substances in exposed, uninoculated media, making them unsuitable for growth.

This exercise demonstrates the toxic action of the ultraviolet rays of the sun on bacteria incorporated in a culture medium. The toxicity may be due to the action of the germicidal rays on bacteria, or on the culture medium, or both.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 72-hr. nutrient broth culture of *Bacillus subtilis*.
3. 2 tubes of nutrient agar.
4. 2 sterile Petri dishes.
5. 2 pieces of black paper.
6. String.

Procedure:

1. Melt two tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

2. Allow to cool to 50°C.

3. Inoculate one tube with two loopfuls of a 24-hr. nutrient broth culture of *E. coli*.

4. Inoculate the other tube with two loopfuls of a 72-hr. nutrient broth culture of *B. subtilis*.

5. Pour the inoculated agar into the Petri dishes.

6. Tilt the dishes from side to side to obtain a uniform distribution of the organisms.

7. Allow the agar to harden.

8. Cut designs, such as initials, in the two pieces of black paper.

9. Remove the lid from one of the dishes and replace it with a piece of the black paper. Tie with a piece of string to hold the paper firmly in place.

10. Do the same to the other plate.

11. Expose the plates to the direct rays of the sun for 3 to 6 hr. without any intervening window glass.

12. Remove the black papers and replace the lids.

13. Incubate the plates at 37°C. for 48 hr.

14. Compare the number and size of the colonies on the exposed areas with those on the darkened areas.

15. Sketch your results.

Questions:

1. Define an angstrom unit.
2. Which are the most germicidal wave lengths of light?
3. Does the depth of the column of the medium play any part in the efficiency of the disinfection process?
4. Why is *B. subtilis* more resistant than *E. coli?*
5. Why should ordinary glass not be placed between the culture and the rays of the sun?
6. What kind of glass may be used?
7. If *E. coli* is suspended in both distilled water and in nutrient broth, in which case would sterilization by light rays be more rapid?   Why?

References

MEDICAL RESEARCH COUNCIL: "A System of Bacteriology," London, vol. 1, 1930.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 28

THE EFFECT OF OSMOTIC PRESSURE

An organism bathed by a solution having an ionic concentration greater than that which exists on the inside of the cell will slowly lose water, resulting in a shrinking of the cellular contents.   This phenomenon is called plasmolysis.   An organism bathed by a solution having an ionic concentration less than that which exists on the inside of the cell will slowly absorb water, resulting in a swelling of the cell.   This phenomenon is called plasmoptysis.

Bacteria are not very sensitive to changes in osmotic pressures. A great increase in the osmotic pressure of the surrounding solution is necessary before any toxic action is noted.   In this respect bacteria differ markedly from higher plant and animal cells.

The purpose of this exercise is to demonstrate the effect of an increase in osmotic pressure on bacteria.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli.*
2. 72-hr. nutrient broth culture of *Bacillus subtilis.*
3. 8 sterile 9-cc. water blanks.
4. 4 sterile test tubes.
5. Sterile 10-cc. pipettes.
6. Sterile 1-cc. pipettes.
7. 6 sterile Petri dishes.

8. 6 tubes of nutrient agar.

9. 30 per cent solution of sodium chloride.

10. 40 per cent solution of sucrose.

Procedure:

1. Pipette 10 cc. of the 40 per cent solution of sucrose into each of two sterile test tubes.

2. Pipette 1 cc. of the 40 per cent solution of sucrose into each of two 9-cc. water blanks. Mix thoroughly.

3. This gives a 4 per cent solution.

4. With the same pipette, remove 1 cc. from each tube of the 4 per cent solution and transfer to two more 9-cc. water blanks. Mix thoroughly.

5. This gives a 0.4 per cent solution.

6. You should now have two series of dilutions of 40 per cent, 4 per cent, and 0.4 per cent sucrose solution.

7. Repeat the same procedure with the 30 per cent solution of sodium chloride to give two series of dilutions of 30 per cent, 3 per cent, and 0.3 per cent sodium chloride solution.

8. Melt six tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

9. Allow the agar to cool to about 50°C.

10. Pour the melted and cooled agar into the six Petri dishes. Observe aseptic precautions.

11. When the agar is firm, invert the plates and divide each into six sectors with a china marking pencil.

12. Arrange the tubes in a block and inoculate from left to right.

13. Inoculate each tube of one set of the sucrose and each tube of one set of the sodium chloride series with a loopful of a 24-hr. nutrient broth culture of *E. coli.*

14. Inoculate each tube of the remaining two sets with one loopful of a 72-hr. nutrient broth culture of *B. subtilis.*

15. Immediately after you have inoculated the last dilution, streak a loopful of the first-treated tube onto the marked sector of the agar plate. Continue with the remaining tubes until all of them have been streaked.

16. Label the plates, invert, and incubate at 37°C. for 48 hr.

17. After a lapse of 1 hr. repeat the operation.

18. Keep the sucrose and sodium chloride dilutions in your desk.

19. After a period of 24 hr., streak the remaining two plates.

20. Record your results in the following table:

SUCROSE

Organisms	Immediately	1 hr.	24 hr.
E. coli			
B. subtilis			

<center>SODIUM CHLORIDE</center>

E. coli

B. subtilis

---

21. Use the following system for indicating the extent of growth:

<center>
0 = no growth.
+ = poor growth.
++ = good growth.
+++ = excellent growth.
</center>

Questions:

    1. Define: (1) plasmolysis, (2) plasmoptysis.

    2. Which of these terms applies to the above effect?

    3. What is a hypertonic solution? A hypotonic solution? An isotonic solution?

    4. Which are more sensitive to osmotic changes, Gram-positive or Gram-negative organisms?

    5. Can an organism adapt itself to growth in a solution of relatively high osmotic pressure?

    6. Give some commercial applications of this phenomenon.

<center>**Reference**</center>

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

<center>EXERCISE 29</center>

<center>**THE EFFECT OF DESICCATION**</center>

Organisms in the dry state do not multiply. Moisture is absolutely necessary for growth. When organisms are dried they gradually die, the rate of death being dependent upon several factors.

The method of desiccation is very important in determining the rate of death of bacteria. If the organisms are dried in a vacuum in the frozen state, the cells may be preserved almost indefinitely. If, on the other hand, the organisms are dried without first being frozen, the protoplasm becomes more and more concentrated as the water evaporates. The cells are then subjected to the plasmolytic action of the salts resulting in an early death.

This exercise demonstrates the effect of drying on vegetative cells and spores.

Required:

    1. 24-hr. nutrient broth culture of *Escherichia coli.*

    2. 72-hr. nutrient broth culture of *Bacillus subtilis.*

    3. 10 sterile Petri dishes.

4. 8 tubes of nutrient agar.

5. 8 clean cover slips.

6. Forceps.

Procedure:

1. Sterilize eight clean cover slips.

2. This may be conveniently carried out by grasping a cover slip near the edge with a pair of forceps and passing several times through the flame of a Bunsen burner. Flame both sides. Too much heat will cause the cover slip to soften and bend.

3. Place four sterilized cover slips in each of two Petri dishes.

4. Transfer a loopful of a 24-hr. nutrient broth culture of *E. coli* to the center of each cover slip of one series.

5. Transfer a loopful of a 72-hr. nutrient broth culture of *B. subtilis* to the center of each cover slip of the other series.

6. Dry the cover slips in the incubator.

7. Handle the cover slips with a pair of forceps previously sterilized in the gas flame.

8. Transfer one cover slip containing the dried culture of *E. coli* to a Petri dish.

9. Transfer one cover slip containing the dried culture of *B. subtilis* to another Petri dish.

10. Melt two tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

11. Allow the agar to cool to a temperature of about 50°C.

12. Pour the melted and cooled agar into the two Petri dishes.

13. Tilt the dishes from side to side to obtain a uniform distribution of the organisms.

14. When the agar has hardened, invert the plates and incubate at 37°C. for 48 hr.

15. Keep the remainder of the prepared cover slips in the 37°C. incubator.

16. Every 48 hr. repeat the above procedure until all the cover slips have been used.

17. Count the colonies that develop on the plates.

18. Record your results in the following table:

Organism	Control	Dried 48 hr.	Dried 96 hr.	Dried 144 hr.
E. coli				
B. subtilis				

Questions:

1. Is there any difference in the number of colonies developing on the two series of plates?

2. Why is *B. subtilis* more resistant to desiccation than *E. coli?*

3. Why are yeasts and molds commonly found in the atmosphere?

4. Do disease organisms live for any considerable length of time when suspended in air? Explain.

5. What effect do capsules have on the resistance of organisms to desiccation?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 30

THE EFFECT OF HYDROGEN-ION CONCENTRATION

Bacteria are sensitive to changes in the hydrogen-ion concentrations of culture media. There exists for every organism an optimum concentration of hydrogen ions at which it will grow best. The pH values above and below at which an organism fails to grow are known as the minimum and maximum hydrogen-ion concentrations, respectively. Variations in such factors as composition of the medium, temperature of incubation, or osmotic pressure of the medium, even though slight, will produce changes in the minimum, optimum and maximum hydrogen-ion values of the organism.

This exercise demonstrates the effect of changes in pH on the growth of an organism.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 14 tubes of nutrient broth each containing 8 cc. of medium.
3. $0.2M$ K_2HPO_4 solution.
4. $0.2M$ boric acid solution.
5. $0.2M$ NaOH solution.
6. $0.1M$ citric acid solution.

Procedure:

1. Prepare the following solutions:

$0.2M$ K_2HPO_4, cc.	$0.1M$ citric acid, cc.	Nutrient broth, cc.	Total, cc.	pH, approximate
0.30	1.70	8	10	2.8
0.60	1.40	8	10	3.6
0.90	1.10	8	10	4.4
1.10	0.90	8	10	5.2
1.30	0.70	8	10	6.0
1.50	0.50	8	10	6.8
1.90	0.10	8	10	7.6

$0.2M$ boric acid, cc.	$0.2M$ NaOH, cc.	Nutrient broth, cc.	Total, cc.	pH, approximate
1.70	0.30	8	10	8.4
1.30	0.70	8	10	9.2
1.00	1.00	8	10	10.0

2. Sterilize all tubes in the Arnold for 20 min. on three successive days.
3. Inoculate each tube with one loopful of a 24-hr. nutrient broth culture of *E. coli.*
4. Incubate the tubes at 37°C. for 48 hr.
5. Examine for turbidity at the end of the incubation period.
6. Record your observations in the following table:

pH	Degree of turbidity	pH	Degree of turbidity
2.8		6.8	
3.6		7.6	
4.4		8.4	
5.2		9.2	
6.0		10.0	

7. Use the following system for indicating the degree of turbidity:

$$0 = \text{no turbidity (clear).}$$
$$+ = \text{slight turbidity.}$$
$$++ = \text{moderate turbidity.}$$
$$+++ = \text{strong turbidity.}$$

Questions:

1. At approximately what pH does maximum turbidity occur?
2. Give the approximate maximum, optimum, and minimum hydrogen-ion concentrations of *E. coli* in the above medium.
3. How could the maximum, optimum, and minimum pH values be changed?
4. Explain the action of buffers.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 31

THE EFFECT OF SURFACE TENSION

Some organisms grow on the surface of the usual culture media in the form of pellicles. These organisms were at one time regarded as

obligate aerobes. It is true that all the usual pellicle-producing bacteria are aerobic but not obligately aerobic. They are capable of growing under both aerobic and anaerobic conditions.

The organisms growing on the surface of a medium are supported in this position by the force of surface tension. If the surface tension is depressed below a certain point and the medium inoculated by a pellicle-producing organism, growth will be diffuse throughout the medium.

This exercise demonstrates the effect of soap as a surface-tension depressant on *Bacillus subtilis*, causing it to grow throughout the medium rather than as a pellicle on the surface.

Required:
1. Sterile 1 per cent solution of sodium ricinoleate in distilled water.
2. 24-hr. nutrient agar slant culture of *Bacillus subtilis*.
3. 3 tubes of nutrient broth.
4. Sterile 1-cc. pipette.

Procedure:
1. Number the three broth tubes with a china marking pencil.
2. Inoculate each tube from a 24-hr. nutrient agar slant culture of *B. subtilis*.
3. Add to each tube the following amount of sodium ricinoleate:
   Tube 1—control
   Tube 2—0.5 cc. of 1 per cent sodium ricinoleate solution.
   Tube 3—0.75 cc. of 1 per cent sodium ricinoleate solution.
4. Mix thoroughly.
5. Incubate the tubes at 37°C. for 24 hr.
6. Examine the character of the growth in each tube.
7. Record the results in your laboratory notebook.

Questions:
1. What is sodium ricinoleate?
2. Is it true that pellicle-producing bacteria are obligate aerobes?
3. What is the precipitate that forms when the soap is mixed with the nutrient broth?
4. How do normal pellicle-forming bacteria differ in composition from the non-pellicle producers?
5. How could you cause an organism to produce a pellicle which it does not do normally?

<div align="center">References</div>

LARSON, W. P.: The Effect of the Surface Tension of the Menstruum upon Bacteria and Toxins. From "The Newer Knowledge of Bacteriology and Immunology," edited by E. O. Jordan and I. S. Falk, Chicago, University of Chicago Press, 1928.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

<div align="center">EXERCISE 32</div>

<div align="center">THE OLIGODYNAMIC ACTION OF HEAVY METALS</div>

The heavy metals in high dilutions are capable of exerting a toxic action on bacteria.

If a silver or copper coin is placed in a Petri dish and covered with melted agar, previously inoculated with an organism, several distinct zones of growth may be noted after an incubation period of 24 hr. Immediately surrounding the coin will be a clear zone in which no colonies appear. This is spoken of as the oligodynamic zone. Beyond this will be a narrower zone in which growth is stimulated. Minute amounts of metallic ions stimulate growth. Normal growth occurs in the remainder of the agar.

This exercise demonstrates the effect of metallic silver or copper on the growth of *Escherichia coli*.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 1 tube of nutrient agar.
3. 2 sterile Petri dishes.
4. Copper or silver coin.
5. 10 per cent solution of nitric acid.
6. Pair of forceps.

Procedure:

1. Clean the coin by immersion in a 10 per cent solution of nitric acid.
2. Wash the coin thoroughly with water to remove the acid.
3. Place the coin in the center of a sterile Petri dish.
4. Melt a tube of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
5. Allow the agar to cool to about 50°C.
6. Inoculate with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
7. Rotate the tube between the palms of the hands to obtain a uniform distribution of organisms.
8. Pour the inoculated agar into the Petri dish containing the coin.
9. Allow the agar to harden.
10. Invert the dish and incubate at 37°C. for 48 hr.
11. Make a drawing of the plate in your notebook.

Questions:

1. Copper exhibits a greater oligodynamic action than silver. Is this due to a greater number of copper ions or to the nature of the ion?
2. Does lead exhibit an oligodynamic action?
3. Does pure silver exhibit an oligodynamic action?
4. Why is the coin washed in nitric acid?
5. Could rubbing of the coin with an abrasive be substituted for the acid treatment?
6. Does the phenomenon of oligodynamic action have any practical application?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

DISINFECTION AND DISINFECTANTS

EXERCISE 33

THE PHENOL COEFFICIENT

The phenol coefficient may be defined as the killing power of a germicide toward a test organism as compared to that of phenol, under identical conditions.

Many modifications of the original method are in use, several of which have been designed, apparently, to favor the compounds being tested. Unless the conditions of the test are specified and adhered to closely, the final results are worthless. Since the compounds to be rated are compared to phenol the final result is spoken of as a phenol coefficient. The organism used in the test must always be named. For example, if the test organism is *Eberthella typhosa*, the final result is spoken of as an *E. typhosa* phenol coefficient.

The procedure followed here is similar to the Food and Drug Administration method, which is standard in this country.

Required:

1. 24-hr. nutrient broth culture of *Staphylococcus aureus*. The organisms are transferred to fresh broth for 5 consecutive days previous to use.
2. 26 tubes of nutrient broth.
3. 13 sterile test tubes.
4. Flask of sterile distilled water.
5. Sterile 5-cc. pipettes.
6. Sterile 10-cc. pipettes.
7. Inoculating loop, 4 mm. outside diameter and made of 24-gauge wire.
8. 5 per cent stock solution of phenol.
9. 5 per cent stock solution of compound solution of cresol.

Procedure:

1. Arrange six sterile tubes in a test-tube block and number them from left to right.
2. Prepare the following dilutions of phenol:

Dilution	1:20 phenol, cc.	Distilled water, cc.	Volume, cc.	Mix; then discard, cc.	Final volume, cc.
1:50	2	3	5	0	5
1:60	2	4	6	1	5
1:70	2	5	7	2	5
1:80	2	6	8	3	5
1:90	2	7	9	4	5
1:100	2	8	10	5	5

3. Pipette 2 cc. of 5 per cent phenol into each of six test tubes.

4. Add to each tube the amount of distilled water specified in the above table.

5. Mix thoroughly.

6. Remove from each tube the amount in excess of 5 cc.

7. In order to avoid the use of too much sterile glassware use one 10-cc. pipette for measuring the 2-cc. portions of phenol and another pipette for preparing the dilutions.

8. Set up another series of tubes and prepare the following dilutions of compound solution of cresol:

Dilutions	1:20 cresol, cc.	Distilled water, cc.	Volume, cc.	Mix; then discard, cc.	Final volume, cc.
1:120	1	5	6	1	5
1:140	1	6	7	2	5
1:160	1	7	8	3	5
1:180	1	8	9	4	5
1:200	1	9	10	5	5
1:220	1	10	11	6	5
1:240	1	11	12	7	5

9. Follow the same procedure for preparing the dilutions as given under phenol.

10. Set up two series of nutrient broth tubes and number the same as the tubes containing the phenol dilutions.

11. Set up two more series of nutrient broth tubes and number the same as the tubes containing the dilutions of compound solution of cresol.

12. At intervals of 30 sec., run 0.5 cc. of a 24-hr. nutrient broth culture of *S. aureus* into each dilution of phenol. Use a 5-cc. pipette and be careful not to contaminate the sides of the tubes with the culture.

13. After the lapse of 5 min. remove a loopful of the mixture from the first dilution and transfer to a tube of nutrient broth.

14. At intervals of 30 sec. remove a loopful from the remaining tubes and transfer to broth medium.

15. After the lapse of another 5 min. repeat the procedure, using the second series of broth tubes.

16. Repeat the foregoing operation with the cresol dilutions in place of the phenol.

17. Incubate all tubes at 37°C. for 48 hr.

18. Examine the tubes for turbidity.

19. Fill in the following table. Report the presence and the absence of growth by + and −.

20. Determine the *S. aureus* phenol coefficient of compound solution of cresol by dividing the greatest dilution of the disinfectant killing the test organism in 10 min. but not in 5 min. by the phenol dilution killing the same organism under identical conditions.

PHENOL

Dilution	5 min.	10 min.
1:50		
1:60		
1:70		
1:80		
1:90		
1:100		

CRESOL

Dilution	5 min.	10 min.
1:120		
1:140		
1:160		
1:180		
1:200		
1:220		
1:240		

Questions:

    1. Is compound solution of cresol a good disinfectant?

    2. What is the trade name of compound solution of cresol?

    3. If *E. coli* were used as the test organism instead of *S. aureus*, would you expect the results to be the same or different?　Explain.

    4. What are some of the objections to rating germicides by their phenol coefficients?

    5. Discuss briefly the limitations of the method.

References

RUEHLE, G. L. A., and C. M. BREWER: United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants, *U. S. Dept. Agr. Circ.* **198,** 1931.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 34

THE SELECTIVE BACTERIOSTATIC ACTION OF CRYSTAL VIOLET

    The triphenylmethane dyes exhibit a pronounced selective bacteriostatic action on Gram-positive organisms in dilutions that have no demonstrable effect on the growth of Gram-negative bacteria.　In general, selective bacteriostatic action parallels the Gram reaction although there are a few notable exceptions.　This means that Gram-positive organisms are more susceptible to the action of these dyes than are the Gram-negative bacteria.

    Crystal violet is a triphenylmethane dye that exhibits an excellent bacteriostatic effect.　In a dilution of 1:10,000 both *Escherichia coli* (Gram −) and *Bacillus subtilis* (Gram +) are inhibited in growth.　In a dilution of 1:100,000 *E. coli* is not affected whereas *B. subtilis* fails to grow.　In a dilution of 1:1,000,000 neither organism is affected and growth proceeds as though no dye were present in the agar medium.

Required:

    1. 24-hr. nutrient broth culture of *Escherichia coli.*

    2. 24-hr. nutrient broth culture of *Bacillus subtilis.*

    3. 1:1000 aqueous solution of crystal violet.

    4. 4 tubes of nutrient agar.

    5. 4 sterile Petri dishes.

    6. Sterile 1-cc. pipette.

Procedure:

    1. Melt four tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

    2. Add to the first tube 1 cc. of a 1:1000 aqueous solution of crystal violet.

    3. Mix thoroughly by rotating the tube between the palms of the hands and at the same time holding the pipette between the fingers.　Use the same pipette for all transfers.

    4. Remove 1 cc. of the agar-dye mixture and transfer to a second tube.

5. Mix in the same manner as before.

6. Remove 1 cc. from the second tube and transfer to the third tube.

7. Pour the contents of the tubes into three separate Petri dishes.

8. Pour the fourth tube of melted agar, without dye, into a Petri dish for a control.

9. When the agar has hardened, invert the plates and divide each into two equal parts with a china marking pencil.

10. Mark each half with the name of the organism to be streaked.

11. Streak one-half of the plate with one loopful of a 24-hr. nutrient broth culture of *B. subtilis* and the other half with one loopful of a 24-hr. nutrient broth culture of *E. coli*.

12. Do likewise with the remaining plates.

13. Incubate the plates in an inverted position at 37°C. for 48 hr.

14. Record your results in the following table.   Record growth by $+$ and absence of growth by $-$.

Dye	Concentration	Growth	
		E. coli	*B. subtilis*
Crystal violet	1:10,000		
	1:100,000		
	1:1,000,000		
Control	No dye		

Questions:

1. Why are dyes referred to as bacteriostatic agents?

2. What is meant by selective bacteriostatic action?

3. Would you expect a spore from a Gram-positive organism to be as susceptible to a bacteriostatic agent as a vegetative cell of the same species?

4. Is the term bacteriostasis synonymous with genesistasis?

5. Do all Gram-positive organisms exhibit the same degree of sensitiveness to bacteriostatic dyes?

6. Is there a perfect correlation between the Gram reaction and bacteriostatic action?

7. Name some practical applications of this phenomenon.

Reference

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE ENZYMES OF BACTERIA

EXERCISE 35

THE HYDROLYSIS OF STARCH

Starch is a complex carbohydrate classed with the polysaccharides.

Some bacteria elaborate an extracellular enzyme known as amylase (diastase), which has the power of hydrolyzing the colloidal starch molecule to maltose. The maltose is diffusible and can enter the bacterial cell where it is attacked by the intracellular or respiratory enzymes.

Bacteria may be placed in two groups on the basis of their action on starch. The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient broth culture of *Bacillus subtilis*.
2. 24-hr. nutrient broth culture of *Escherichia coli*.
3. 2 sterile Seitz or Berkefeld type filters.
4. 2 sterile 500-cc. filter flasks.
5. 2 flasks of starch broth each containing 250 cc. of medium.
6. 3 tubes of starch broth each containing 5 cc. of medium.
7. 2 tubes of starch agar.
8. 2 sterile Petri dishes.
9. Iodine solution.

Procedure:

IN THE ABSENCE OF BACTERIA

1. Inoculate a flask of starch broth with one loopful of a 24-hr. nutrient broth culture of *B. subtilis*.
2. Inoculate a second flask with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
3. Incubate both flasks at a temperature of 37°C. for five days.
4. Filter the culture of *B. subtilis* through a sterile Seitz or Berkefeld type filter and collect the filtrate in a sterile 500-cc. filter flask.
5. Do likewise with the starch broth culture of *E. coli*.[1]
6. Add 3 cc. of the *B. subtilis* filtrate to a tube of starch broth.
7. Add 3 cc. of the *E. coli* filtrate to a second tube of starch broth.
8. The third tube is kept as a control.
9. Incubate all tubes at 37°C. for 24 hr.
10. At the end of the incubation period add a few drops of iodine solution to each tube and shake. Note color.

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[1] The instructor should inoculate the flasks and prepare the filtrates. The quantities given should be sufficient for a class of 50 students.

In the Presence of Bacteria

1. Melt two tubes of starch agar in an Arnold sterilizer or in a pan of boiling water.

2. Allow the agar to cool to about 50°C.

3. Pour the melted and cooled agar into two sterile Petri dishes.

4. Set the plates aside until the agar is firm.

5. Streak over the surface of one plate one loopful of a 24-hr. nutrient broth culture of *B. subtilis*.

6. Streak over the surface of the other plate one loopful of a 24-hr. nutrient broth culture of *E. coli*.

7. Incubate the plates at 37°C. for 48 hr.

8. At the end of the incubation period flood the surface of the agar with a dilute solution of iodine.

9. Note the color produced.

10. Record your observations in the following table:

Organism	Hydrolysis of starch	
	Filtrate	Culture
B. subtilis		
E. coli		

Interpret your results in both experiments.

Questions:

1. Why is iodine added?

2. Why is a blue color produced under some conditions?

3. Why does one of the plates remain colorless?

4. Could all the enzymes produced by bacteria be obtained by the above method of filtration? Explain.

5. Why is starch broth used instead of nutrient broth for preparing the culture filtrates?

6. What does the exercise demonstrate?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 36

THE LIQUEFACTION OF GELATIN

Gelatin is a protein prepared by the hydrolysis of the insoluble protein collagen.

It produces a jelly when dissolved in water. Since it is a protein, it can be attacked by many organisms, resulting in a loss of its property to jell. The enzyme responsible for this action is known as a gelatinase. It is an extracellular enzyme and its presence may be identified by adding some culture filtrate to the appropriate substrate. In the presence of carbohydrates that are rapidly fermented the enzyme is usually not produced. Therefore, noncarbohydrate media should be used to demonstrate the ability of an organism to elaborate a gelatinase.

Some organisms elaborate a gelatinase and others do not. The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient gelatin culture of *Proteus vulgaris.*
2. 24-hr. nutrient gelatin culture of *Escherichia coli.*
3. 3 tubes of nutrient gelatin.
4. 5 per cent aqueous solution of phenol.

Procedure:

IN THE PRESENCE OF LIVING BACTERIA

1. Inoculate a tube of nutrient gelatin with one loopful of a 24-hr. gelatin culture of *P. vulgaris.*
2. Inoculate a second tube with one loopful of a 24-hr. gelatin culture of *E. coli.*
3. Incubate the tubes at 37°C. for 48 hr.
4. Place the tubes in the refrigerator and allow them to remain for about 1 hr.
5. Does the gelatin harden?

IN THE ABSENCE OF LIVING BACTERIA

1. Melt a 10-cc. tube of nutrient gelatin in an Arnold sterilizer or in a pan of boiling water.
2. Add to the melted gelatin 1 cc. of a 5 per cent aqueous solution of phenol.
3. This gives a final phenol concentration of about 0.5 per cent.
4. Rotate the tube between the palms of the hands to obtain a uniform mixture of phenol and medium.
5. Allow the tube to cool to about 45°C.
6. Add to the melted and cooled gelatin 0.5 cc. of a 24-hr. nutrient gelatin culture of *P. vulgaris.*
7. Again rotate the tube.
8. Incubate at 37°C. for 48 hr.
9. Remove the tube from the incubator and place in the refrigerator. Allow to remain for 1 hr.
10. Does the gelatin harden?
11. Record your observations in the following table:

| Organism | Liquefaction of gelatin | |
	In the presence of living bacteria	In the absence of living bacteria
P. vulgaris		
E. coli.		

Interpret your results in both experiments.

Questions:

1. Why is gelatin medium cooled to a temperature of 45°C. before adding the culture of *P. vulgaris?*

2. Why does *E. coli* fail to liquefy gelatin?

3. Why is phenol added?

4. Are enzymes more resistant to the action of germicides than the cells producing them?

5. What effect does the presence of a fermentable carbohydrate usually have on gelatinase production? Why?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 37

THE HYDROLYSIS OF CASEIN

Casein is a phosphoprotein and is the principal nitrogenous constituent of milk.

Some organisms are able to hydrolyze the protein and convert it into soluble compounds. The conversion of casein into a mixture of soluble compounds is sometimes spoken of as peptonization.

The enzyme responsible for the hydrolysis of casein is called a casease. It is an example of an extracellular enzyme. The presence of the enzyme may be determined by inoculating the surface of milk agar with a culture of the organism being studied. The elaboration of a casease is noted by the appearance of clear zones surrounding the colonies.

Some organisms elaborate a casease and others do not. The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient broth culture of *Bacillus subtilis.*

2. 24-hr. nutrient broth culture of *Escherichia coli.*

3. 24-hr. nutrient broth culture of *Streptococcus lactis.*

4. 3 tubes of nutrient agar.
5. 1 tube of sterile milk.
6. 3 sterile Petri dishes.

Procedure:

1. Melt three tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

2. Allow the agar to cool to a temperature of about 50°C.

3. Pipette about 2 cc. of sterile milk into each of three Petri dishes.

4. Pour the melted and cooled agar into the plates.

5. Tilt the dishes from side to side to obtain a uniform mixture of milk and nutrient agar.

6. Set the plates aside until the agar is firm.

7. Streak over the surface of one plate one loopful of a 24-hr. nutrient broth culture of *B. subtilis*.

8. Streak over the surface of a second plate one loopful of a 24-hr. nutrient broth culture of *E. coli*.

9. Streak over the surface of the last plate one loopful of a 24-hr. nutrient broth culture of *S. lactis*.

10. Invert the plates and incubate at 37°C. for 96 hr.

11. Examine the plates for the presence of clear zones surrounding the colonies.

12. Note the odor given off from the surface of the plates.

13. Record your observations in the following table:

Organism	Peptonization	Odor	
		Fermentative	Putrefactive
B. subtilis			
E. coli			
S. lactis			

Questions:

1. What is peptonized milk?

2. Why is *E. coli* unable to peptonize milk?

3. Is the peptonization of casein increased or decreased in the presence of a rapidly fermenting organism? Why?

4. Is casease an intra- or an extracellular enzyme? How can you tell by looking at your plates?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE RESPIRATION OF BACTERIA

EXERCISE 38

THE CULTIVATION OF ANAEROBIC BACTERIA

Anaerobes are organisms that grow only in a greatly reduced oxygen environment. They show great variation in the amount of free oxygen they are able to tolerate. Some are able to grow in the presence of relatively large amounts of free oxygen; others cannot grow unless the conditions are almost anaerobic.

Many methods are employed for the cultivation of anaerobic bacteria. They can all be placed in two groups on the basis of the mechanisms involved: (1) increasing the reducing intensity of the medium and (2) reducing the oxidizing activity of the atmosphere.

Required:

1. 24-hr. nutrient broth culture of *Serratia marcescens*.
2. 48-hr. glucose brain culture of *Clostridium sporogenes*.
3. 1 tube of thioglycollate broth.
4. 1 tube of deep glucose broth.
5. 1 tube of deep nutrient broth.
6. 1 tube of glucose brain medium.
7. 1 glucose blood agar plate or glucose agar plate.
8. Sterile 1-cc. pipettes.
9. Vaspar.
10. Plasticine.
11. Slides.

Procedure:

INCREASING THE REDUCING INTENSITY OF THE MEDIUM

Addition of Sodium Thioglycollate. 1. Place a tube of sodium thioglycollate broth in an Arnold sterilizer and steam for 10 min.

2. Remove the tube from the sterilizer and allow to cool to a temperature of 50°C.

3. Inoculate the tube with one loopful of a 48-hr. glucose brain culture of *C. sporogenes*.

4. Incubate the tube at 37°C. for 24 to 48 hr.

5. Prepare a Gram stain and examine the slide under the oil-immersion objective.

Addition of Dead Tissue. 1. Place a tube of glucose brain medium in an Arnold sterilizer and steam for 10 min.

2. Remove the tube from the Arnold and allow to cool to a temperature of 50°C.

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3. Inoculate the tube deep with *C. sporogenes* using a wire loop or 1-cc. pipette.

4. Incubate the tube at 37°C. for 24 to 48 hr.

5. The organisms begin to multiply in the deeper layers of the medium. The glucose is rapidly fermented with the production of acid and gas. The escaping stream of gas produces anaerobic conditions throughout the medium.

6. Prepare a Gram stain and examine the slide under the oil-immersion objective.

Addition of Aerobic Bacteria (Fortner Method). 1. Obtain a plate containing glucose blood agar or glucose agar. The former is preferable.

2. Examine the surface of the agar for droplets of condensed moisture. If moisture is present, the plate must be incubated until the surface is dry.

3. Divide the plate into two equal parts by making a heavy line on the bottom of the dish with a china marking pencil.

4. Streak one-half of the plate with one loopful of a 48-hr. glucose brain culture of *C. sporogenes.*

5. Streak the other half with one loopful of a 24-hr. nutrient broth culture of *S. marcescens.*

6. Carefully seal the edges of the cover and dish with plasticine clay to prevent the entrance of atmospheric oxygen.

7. Invert the plate and incubate at 37°C. for 48 hr.

8. Examine the surface for colonies of *C. sporogenes.*

9. Prepare a Gram stain and examine under the oil-immersion objective.

REDUCING THE OXIDIZING ACTIVITY OF THE ATMOSPHERE

Exclusion of Atmospheric Oxygen. 1. Place one tube of deep nutrient broth and one of deep glucose broth in an Arnold sterilizer and steam for 10 min.

2. Remove the tubes from the Arnold and allow to cool to a temperature of 50°C.

3. Inoculate the tubes deep with *C. sporogenes*, using a wire loop or 1-cc. pipette.

4. Cover the surface of the media with a ½-in. layer of melted vaspar (a mixture of equal parts of vaseline and paraffin).

5. Incubate the tubes at 37°C. for 24 to 48 hr.

6. The glucose in the carbohydrate medium is fermented with the production of acid and gas, with the result that the vaspar seal is forced up toward the neck of the tube. In some cases the cotton plug and seal are expelled completely from the tube.

7. Prepare Gram stains and examine the slides under the oil-immersion objective.

8. Record all results in your notebook.

Questions:

1. What is meant by anaerobiosis?

2. Why is a broth medium with carbohydrate more satisfactory than one without it for the cultivation of anaerobes?

3. Is *C. sporogenes* killed at a temperature of 50°C.?

4. How does brain medium create anaerobic conditions favorable to the growth of *C. sporogenes?*

5. Why is glucose blood agar more satisfactory than glucose agar in the Fortner plate method?

6. Why should the Fortner plate be free from condensed moisture before inoculation?

7. Why is a small amount of agar present in the thioglycollate broth?

References

BREWER, J. H.: A Clear Liquid Medium for the "Aerobic" Cultivation of Anaerobes, *J. Bact.*, **39**: 10, 1940.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 39·

THE REDUCTION OF METHYLENE BLUE

Methylene blue acts as a hydrogen acceptor and becomes decolorized. The speed of decolorization of the dye is an indication of the rate at which oxidation takes place. If air or oxygen is bubbled through the medium containing the reduced dye, the blue color is restored.

Methylene blue added to cultures of bacteria is quickly decolorized because the organisms contain organic substances that are activated by certain enzymes known as dehydrogenases. A dehydrogenase may be defined as an intracellular enzyme that is capable of activating the hydrogen of metabolites so that they can be oxidized aerobically in the presence of oxygen or anaerobically in the presence of a suitable reducible substance.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli.*
2. 1:20,000 aqueous solution of methylene blue
3. 50 cc. nutrient broth.
4. 10-cc. pipettes.
5. 1-cc. pipettes.
6. 5 sterile test tubes, ⅝ by 6 in.

Procedure:

1. Prepare the following dilutions of a 24-hr. nutrient broth culture of *E. coli:*

Tube no.	Nutrient broth, cc.	Culture of *E. coli*, cc.	Total volume, cc.
1	9	1	10 ·
2	8	2	10
3	7	3	10
4	6	4	10
5	5	5	10

2. Add to each tube 1 cc. of the solution of methylene blue.
3. Mix thoroughly by means of a sterile 1-cc. pipette.
4. Incubate the tubes at 37°C.
5. Examine at 30-min. intervals until decolorization is complete, except in the surface layer.
6. Record your observations in the following table:

Tube no.	Decolorization time

7. Shake the decolorized tubes vigorously for a few seconds. Does the blue color return?

Questions:

1. What is a hydrogen acceptor?
2. Does methylene blue act as a hydrogen acceptor?
3. What is the purpose of the reaction?
4. Where does decolorization begin first? Why?
5. Which enzymes are responsible for the decolorization of methylene blue?
6. Why is the blue color restored to the decolorized tubes after vigorous shaking?
7. Does the phenomenon have any practical application? (See page 118.)

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 40

THE REDUCTION OF NITRATES

Many organisms are capable of reducing nitrates to nitrites. Some can reduce the nitrites to ammonia. Still others are not able to attack nitrates but can reduce nitrites to ammonia. The nitrates and nitrites support anaerobic growth by acting as hydrogen acceptors.

The reduction of nitrates and nitrites proceeds more rapidly in the presence of an anaerobic environment. If a culture is well-aerated, reduction does not occur. The reaction of the medium is of importance in determining the extent of reduction. In an alkaline medium the nitrates are reduced to nitrites whereas in an acid environment the reaction may proceed to the ammonia stage.

The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient agar slant culture of *Proteus vulgaris*.
2. 24-hr. nutrient agar slant culture of *Pseudomonas fluorescens*.

3. 3 tubes of nitrate broth.
4. Sulfanilic acid test solution.
5. α-Naphthylamine test solution.
6. Test tubes.
7. Two 1-cc. pipettes.

Procedure:

1. Inoculate a tube of nitrate broth from a 24-hr. nutrient agar slant culture of *P. vulgaris.*

2. Inoculate a second tube from a 24-hr. nutrient agar slant culture of *Ps. fluorescens.*

3. Keep the third tube for a control.

4. Incubate the tubes at a temperature of 37°C.

5. Test for nitrate reduction by removing aseptically 1 cc. of the broth from the two inoculated tubes and the control and transferring to test tubes. Add to each tube 2 drops of sulfanilic acid reagent and 2 drops of α-naphthylamine solution. The presence of nitrite is indicated by a pink or red color.

6. Record your results in the following table:

REDUCTION OF NITRATE TO NITRITE

Organism	Days									
	2	4	6	8	10	12	14	16	18	20
P. vulgaris										
Ps. fluorescens										
Control										

7. Continue to incubate the tubes until one of the cultures shows a negative nitrite test. This indicates that the nitrite has been reduced to ammonia.

8. Record your results in the following table:

REDUCTION OF NITRITE TO AMMONIA

Organism	Days									
	2	4	6	8	10	12	14	16	18	20
P. vulgaris										
Ps. fluorescens										
Control										

Questions:
1. How is sulfanilic acid solution prepared?
2. How is α-naphthylamine solution prepared?
3. Why do organisms reduce nitrates?
4. Under what conditions are nitrates reduced?
5. What does a negative nitrite test mean?

References

Committee on Bacteriological Technic: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1939.

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 41

THE REDUCTION OF HYDROGEN PEROXIDE

Catalase is an enzyme capable of decomposing hydrogen peroxide into water and molecular oxygen.

Catalase is produced by many bacteria. It is present in greatest amounts in the strictly aerobic bacteria. The enzyme is not produced by the obligately anaerobic bacteria.

The presence of the enzyme may be demonstrated by adding hydrogen peroxide to a culture and noting the evolution of oxygen. In the absence of the enzyme no appreciable decomposition of the peroxide occurs.

Required:
1. 24-hr. nutrient broth culture of *Escherichia coli.*
2. 24-hr. nutrient broth culture of *Staphylococcus aureus.*
3. 24-hr. nutrient broth culture of *Bacillus subtilis.*
4. 24-hr. nutrient broth culture of *Proteus vulgaris.*
5. 5 Smith fermentation tubes containing nutrient broth.
6. Solution of hydrogen peroxide.
7. 1-cc. pipette.

Procedure:
1. Inoculate a Smith fermentation tube with one loopful of a 24-hr. nutrient broth culture of *E. coli.*
2. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *S. aureus.*
3. Inoculate a third tube with one loopful of a 24-hr. nutrient broth culture of *B. subtilis.*
4. Inoculate a fourth tube with one loopful of a 24-hr. nutrient broth culture of *P. vulgaris.*
5. The fifth tube serves as a control.
6. Incubate the tubes at 37°C. for 48 hr.
7. At the end of the incubation period add 1 cc. of a solution of hydrogen peroxide to each of the tubes.
8. This is best added by introducing the tip of a 1-cc. pipette containing peroxide into the fermentation tube as far as it will go, then removing the finger.
9. Allow the liquid to run in without blowing on the pipette to prevent the introduction of any air.

10. Allow the tubes to stand for about 1 hr.

11. Measure the per cent of gas evolved in the closed arm of the tubes by means of a Frost gasometer (Fig. 3) and record the results in the following table:

	Organism				
	E. coli	*S. aureus*	*B. subtilis*	*P. vulgaris*	Control
Per cent O_2 evolved					

Questions:

1. What is the nature of catalase?
2. How does it differ from peroxidase?
3. How do catalase and peroxidase differ in their functions?
4. Why is a piece of living plant or animal tissue able to decompose hydrogen peroxide?
5. Is it true that only free-oxygen-requiring cells contain catalase?
6. Is catalase an intracellular or an extracellular enzyme?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 42

THE PRODUCTION OF PIGMENTS BY BACTERIA

Many bacteria are able to produce colored compounds known as pigments. These compounds must be distinguished from the photosynthetic pigments produced by some sulfur bacteria that are capable of converting carbon dioxide into organic matter in the presence of light under anaerobic conditions.

Some pigments remain confined inside of the bacterial cells; others are secreted into the culture media. Only a few of the pigments are water soluble; most of them are soluble in fat solvents such as ether, chloroform, acetone, carbon disulfide, or alcohol.

Pigments are probably produced under both aerobic and anaerobic conditions but the colored form usually appears only in the presence of free oxygen. Pigmented cultures placed under anaerobic conditions gradually lose their characteristic color. When such cultures are again exposed to air, the color gradually returns.

The exact function of bacterial pigments is not known. Their presence is made use of in identifying and classifying bacteria.

Required:

1. Glucose yeast extract agar slant culture of *Serratia marcescens*.
2. Glucose yeast extract agar slant culture of *Rhodococcus roseus*.
3. Glucose yeast extract agar slant culture of *Sarcina lutea*.
4. Glucose yeast extract agar slant culture of *Pseudomonas fluorescens*.
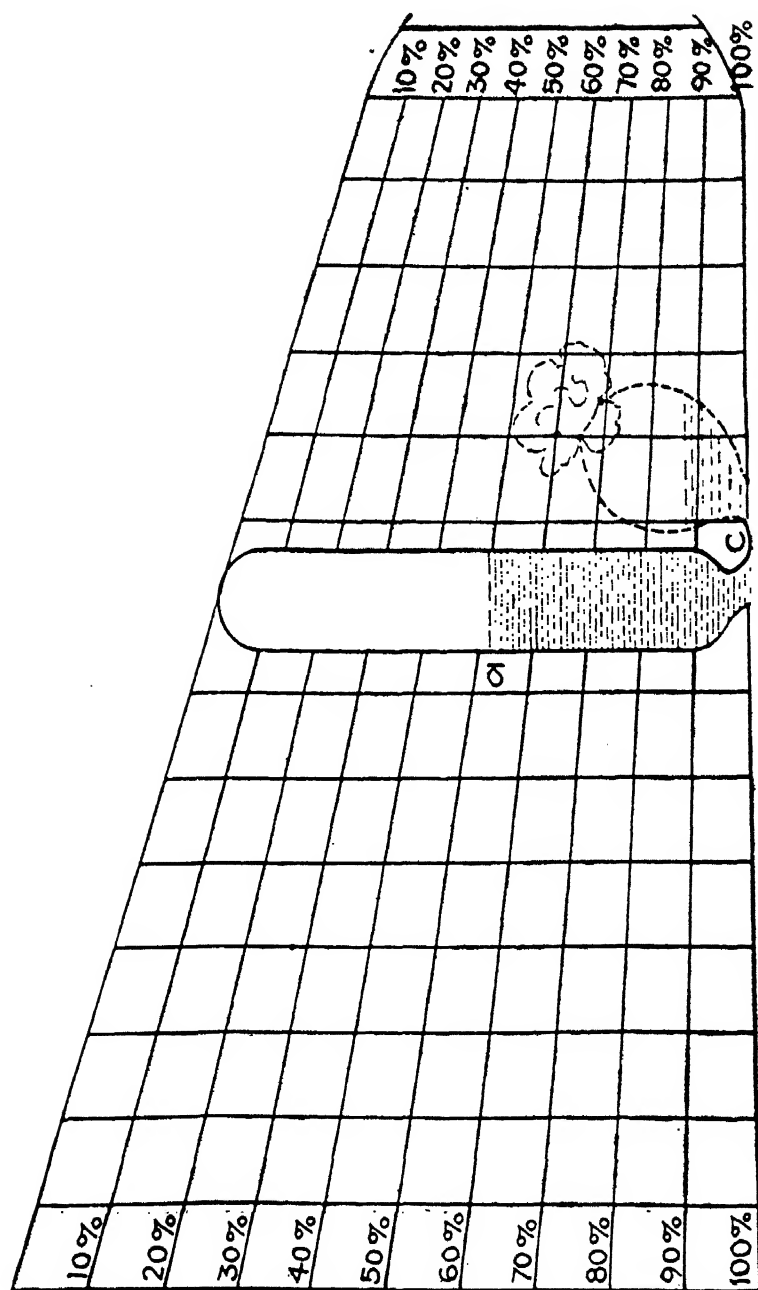
FIG. 3.—Frost gasometer. Tear out and use for measuring the percentage of gas in Smith fermentation tubes. (From Heinemann, "Laboratory Guide in Bacteriology," University of Chicago Press.)

5. Glucose yeast extract agar slant culture of *Chromobacterium violaceum.*
6. 10 sterile glucose yeast extract agar slants.
7. 25 clean test tubes with cork stoppers.
8. Alcohol.
9. Ether.
10. Chloroform.
11. Acetone.

Procedure:

1. Prepare two glucose yeast extract agar slant cultures of each organism.
2. Place one series in your locker; the other in the 37°C. incubator.
3. Incubate the tubes for 1 week or until good pigment production occurs.
4. Place a few drops of the different solvents in each of five test tubes.
5. Remove small amounts of the bacterial growth with the wire loop and emulsify in the solvents.
6. Cork the tubes securely and shake vigorously.
7. Do likewise with the remaining cultures.
8. Record your results in the following table:

Organism	Presence and color of pigment at		Diffusibility of pigment in agar	Solubility of pigment*				
	20°C.	37°C.		W	A	E	C	Ac
S. marcescens								
R. roseus								
S. lutea								
Ps. fluorescens								
C. violaceum								

*W = water; A = alcohol; E = ether; C = chloroform; Ac = acetone.

Questions:

1. Why is glucose yeast extract agar preferred for good pigment production?
2. Why do most pigmented cultures lose their color under anaerobic conditions?
3. Where are most pigmented organisms found in nature?

4. Why is a temperature of 25°C. generally more suitable for good pigment production than 37°C.?

References

ANDERSON, C. G.: "An Introduction to Bacteriological Chemistry," Baltimore, William Wood & Company, 1938.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE DECOMPOSITION AND PUTREFACTION OF PROTEINS

EXERCISE 43

THE PRODUCTION OF INDOLE

Indole is a putrefactive compound produced by the action of some bacteria on the amino acid tryptophane. Since tryptophane is the only naturally occurring amino acid containing the indole ring, the test is specific for this compound.

Tryptophane is attacked by bacteria in different ways. Not all organisms are capable of degrading the amino acid to indole. The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 24-hr. nutrient broth culture of *Proteus vulgaris*.
3. 24-hr. nutrient broth culture of *Staphylococcus aureus*.
4. 9 tubes of tryptone broth.
5. Ehrlich-Böhme reagents 1 and 2.
6. Gnezda oxalic acid test papers.

Procedure:

1. Inoculate three tubes of tryptone (tryptophane) broth each with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
2. Inoculate three more tubes of tryptone broth each with one loopful of a 24-hr. nutrient broth culture of *P. vulgaris*.
3. Inoculate the remaining three tubes of tryptone broth each with one loopful of a 24-hr. nutrient broth culture of *S. aureus*.
4. Into one tube of each set suspend strips of oxalic acid test papers. These may be held firmly in position by placing the papers against the inside surfaces of the tubes and replacing the cotton stoppers.
5. Incubate the cultures at 37°C. for four days. Two days are ordinarily sufficient if good growth is obtained.
6. At the end of the incubation period complete the tests as outlined below.

GNEZDA OXALIC ACID METHOD

7. Examine the three tubes containing the suspended strips of oxalic acid paper. If indole is formed, the oxalic acid crystals are colored pink.
8. Indole is a volatile compound so, if any is produced by the organisms under examination, the papers will be colored pink. This is the only volatile compound

elaborated by bacteria capable of producing a pink color with oxalic acid crystals. Therefore, the test is specific for indole.

EHRLICH-BÖHME METHOD

9. Add to each tube of the second set of cultures about 5 cc. of solution 1 followed by about 5 cc. of solution 2 and shake thoroughly.

10. A red color appears within 5 min. if the test is positive.

11. This color reaction is positive not only for indole but also for α-methylindole. Therefore the test is not so specific as the Gnezda oxalic acid method.

GORÉ METHOD

12. Goré modified the method of Ehrlich-Böhme, making it specific for indole only.

13. Remove the cotton stoppers from the tubes of the remaining set of cultures.

14. Moisten each stopper with 6 drops of solution 2 followed by 6 drops of solution 1. White absorbent cotton must be used for the stoppers, otherwise the results will be unsatisfactory.

15. Replace the stoppers and force them down into the tubes until the moistened ends are about $1\frac{1}{2}$ in. above the surfaces of the cultures.

16. Place the tubes in a boiling water bath and continue the heat for about 15 min., being careful not to allow the cultures to come in contact with the treated stoppers.

17. The appearance of a red color indicates the presence of indole.

18. Record your results as $+$ or $-$ in the following table:

Organism	Indole reaction		
	Ehrlich–Böhme	Gnezda	Goré
E. coli			
P. vulgaris			
S. aureus			

Questions:

1. How are the Ehrlich-Böhme reagents prepared?
2. How are the Gnezda oxalic acid test papers prepared?
3. What does a negative indole test mean?
4. Why is the Ehrlich-Böhme test less specific than the Gnezda test?
5. Could indole tests be performed on agar slant cultures?
6. Does any other naturally occurring amino acid contain the indole ring?
7. Do all proteins contain tryptophane?

References

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 44

THE EFFECT OF CARBOHYDRATE ON INDOLE PRODUCTION

In the absence of a fermentable carbohydrate bacteria utilize the amino acids of peptone for both structure and energy. Many products of decomposition and putrefaction are produced from the intracellular utilization of the amino acids.

In the presence of a fermentable carbohydrate that is rapidly attacked with the production of a considerable amount of acid in a short period of time, activity is inhibited before the organisms are able to elaborate sufficient proteolytic enzymes to attack the nitrogenous constituents of the medium. Under these conditions a pronounced protein-sparing action occurs.

On the other hand, organisms that ferment carbohydrates very slowly or those that do not produce strongly acid products fail to show a protein-sparing action. Protein breakdown occurs as though there were no carbohydrate present.

If tests for indole are to be made, it is best to employ noncarbohydrate media, otherwise misleading conclusions may be drawn.

Required:
1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 24-hr. nutrient broth culture of *Escherichia communior*.
3. 6 tubes of tryptone broth.
4. 6 tubes of sucrose broth.
5. Gnezda oxalic acid test papers.
6. Ehrlich-Böhme reagents 1 and 2.

Procedure:
1. Inoculate three tubes of tryptone broth and three tubes of sucrose broth each with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
2. Inoculate three tubes of tryptone broth and three tubes of sucrose broth each with one loopful of a 24-hr. nutrient broth culture of *E. communior*.
3. Into one set of the tubes suspend strips of oxalic acid test papers. These may be held firmly in position by placing the papers against the inside surfaces of the tubes and replacing the cotton stoppers.
4. Incubate all tubes at 37°C. for 4 days. Two days of incubation is ordinarily sufficient if good growth occurs.
5. At the end of the incubation period complete the tests as outlined under Exercise 43 (page 81).
6. Record your results in the following table:

Organism	Medium	Indole reaction		
		Ehrlich-Böhme	Gnezda	Goré
E. coli	Tryptone broth			
	Sucrose broth			
E. communior	Tryptone broth			
	Sucrose broth			

Questions:

    1. Why is tryptone broth recommended for the indole test?

    2. Are both colon organisms capable of fermenting sucrose?

    3. How do you explain the different indole results of the two organisms inoculated in sucrose broth?

    4. What general conclusions can you draw from this experiment?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 45

THE PRODUCTION OF AMMONIA

The accumulation of ammonia in bacterial cultures usually results from the deaminization of amino acids. It is believed that amino acids must be first deaminized prior to utilization.

In the absence of a protein-sparing action, amino acids are used for both structure and energy. Since more carbon is required for energy than nitrogen for structure, ammonia will accumulate in the medium. In the presence of a fermentable carbohydrate a protein-sparing action may occur, in which case the organisms utilize the amino acids for structure but not for energy. Under these conditions less free ammonia will accumulate than in the same medium without carbohydrate.

The formation of ammonia in cultures is a general reaction performed by any organism capable of utilizing amino acids or similar compounds for structural purposes.

Required:

    1. 24-hr. nutrient broth culture of *Escherichia coli.*
    2. 24-hr. nutrient broth culture of *Proteus vulgaris.*
    3. 24-hr. nutrient broth culture of *Staphylococcus aureus.*
    4. 24-hr. nutrient broth culture of *Bacillus subtilis.*
    5. 5 tubes of nutrient broth of pH7.2.
    6. 5 strips of red litmus paper.

Procedure:

    1. Inoculate a tube of nutrient broth with one loopful of a 24-hr. nutrient broth culture of *E. coli.*

    2. Inoculate a second tube of nutrient broth with one loopful of a 24-hr. nutrient broth culture of *P. vulgaris.*

    3. Inoculate a third tube of nutrient broth with one loopful of a 24-hr. nutrient broth culture of *S. aureus.*

    4. Inoculate a fourth tube of nutrient broth with one loopful of a 24-hr. nutrient broth culture of *B. subtilis.*

    5. The fifth tube serves as a control.

    6. Incubate all tubes at 37°C. for 2 days.

    7. Remove the cotton stopper from one of the incubated cultures and place a strip of red litmus paper against the inside wall of the tube. Replace the cotton stopper to hold the litmus strip in place. Do not allow the broth to touch the litmus paper.

    8. Do likewise with the other four tubes.

    9. Place the tubes, including the control, in a pan or beaker of boiling water and continue the heat for a period of about 5 min.

    10. Do the litmus strips change in color?

    11. Record your results in the following table:

Organisms	Color of litmus paper
Control	
S. aureus	
P. vulgaris	
B. subtilis	
E. coli	

Questions:

    1. What is meant by a protein-sparing reaction?

    2. Why does carbohydrate spare protein?

    3. What effect would the presence of glucose have on ammonia production by *E. coli?* By *B. subtilis?* Explain.

    4. In the presence of a fermentable carbohydrate, under what conditions do you get a fermentation? A putrefaction?

References

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

STEPHENSON, M.: "Bacterial Metabolism," New York, Longmans, Green and Company, 1939.

EXERCISE 46

THE PEPTONIZATION AND FERMENTATION OF MILK

Milk is used as a differential medium to demonstrate the ability of an organism to produce a fermentation, or a peptonization, or a simultaneous fermentation and peptonization.

A rapid utilization of the lactose with the formation of considerable acid results in a fermentative reaction. The casein is curdled and is accompanied by the separation of a clear liquid known as whey. The reaction is strongly acid.

A weak action on the lactose or none at all results in a peptonization. This is usually preceded by formation of a soft coagulum known as a rennin curd. The casein becomes peptonized or decomposed with the formation of various soluble products. The reaction is usually alkaline.

The vegetable indicator litmus is frequently added to milk to ascertain acid production and also the ability of an organism to reduce the compound to its colorless form.

The changes that some organisms produce in milk are used for their identification and classification.

Required:

    1. 24-hr. nutrient broth culture of *Escherichia coli.*

    2. 24-hr. nutrient broth culture of *Bacillus subtilis.*

    3. 2 tubes of litmus milk.

Procedure:

    1. Inoculate a tube of litmus milk with one loopful of a 24-hr. nutrient broth culture of *E. coli*.

    2. Inoculate a second tube of litmus milk with one loopful of a 24-hr. nutrient broth culture of *B. subtilis.*

    3. Incubate both tubes at 37°C. for 48 hr.

    4. Record your observations in the following table:

| Organism | Reaction | Curd | | Peptoni-zation | Reduction of litmus | |
		Acid	Rennin		Begins in, days	Ends in, days
E. coli						
B. subtilis						

Questions:

1. Why do many organisms multiply very rapidly in milk?
2. How would you distinguish a rennin curd from an acid curd?
3. What is whey?
4. Does *B. subtilis* produce a rennin curd?
5. Does the action of *B. subtilis* result in the formation of whey? Why?
6. What is meant by peptonization?
7. Where does the decolorization of the litmus occur first? Explain.
8. Why is the indicator decolorized?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 47

THE PRODUCTION OF HYDROGEN SULFIDE

The sulfur-containing amino acid cystine is dissimilated by some organisms with the liberation of hydrogen sulfide. This is considered a putrefactive compound.

Heavy metal salts are usually incorporated in culture media to detect hydrogen sulfide production. The metals react with the hydrogen sulfide as it is formed to give metal sulfides. Since these compounds are colored, their presence can be easily detected.

Lead has been used more than any other heavy metal for this purpose. Since it is not very sensitive, it is being slowly replaced by other heavy metals, especially iron, nickel, cobalt, and bismuth.

Some organisms are capable of degrading cystine to hydrogen sulfide whereas others are not. The test is of value in identifying and classifying bacteria.

Required:

1. 24-hr. nutrient agar slant culture of *Proteus vulgaris*.
2. 24-hr. nutrient agar slant culture of *Escherichia coli*.
3. 2 tubes of cobalt-nickel agar medium.

Procedure:

1. Remove some of the growth from a 24-hr. nutrient agar slant culture of *P. vulgaris* with the wire needle and inoculate a tube of cobalt-nickel agar by stabbing the medium.

2. In like manner inoculate a second tube from a 24-hr. nutrient agar slant culture of *E. coli.*

3. Incubate the tubes at 37°C. for 2 to 4 days.

4. Examine the tubes for any darkening along the line of growth.

5. Check the purity of the cultures by preparing Gram stains.

6. Record your results in the following table:

Organism	Black color		Gram reaction	Composition of black compound
	Light	Heavy		
E. coli				
P. vulgaris				

Questions:

1. How is cobalt-nickel agar medium prepared?

2. Can you write equations for the reactions that occur?

3. If gelatin were added as the only nitrogen source, would you obtain a positive sulfide test?

4. Is the reaction aerobic or anaerobic?

5. Can organisms utilize hydrogen sulfide as a source of energy?

References

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

UTERMOHLEN, W. P., JR., and C. E. GEORGI: A Comparison of Cobalt and Nickel Salts with Other Agents for the Detection of Hydrogen Sulfide in Bacterial Cultures, *J. Bact.*, **40**: 449, 1940.

THE FERMENTATION OF CARBOHYDRATES
AND RELATED COMPOUNDS

EXERCISE 48

THE FERMENTATION OF CARBOHYDRATES

Organisms vary considerably in their ability to attack and ferment various carbohydrates. Some organisms are able to attack a carbohydrate and produce acid and gas; others are able to produce acid but no gas; still others fail to ferment the compound. Such information is of considerable value in the identification and classification of organisms.

It is not clearly understood why an organism ferments one sugar and not another having the same empirical formula. The sugars differ only in the arrangement of H atoms and OH groups around carbon atoms. There is no method for determining beforehand if an organism is capable of fermenting a given carbohydrate. This can be determined only by making the test.

Required:

  1. 24-hr. nutrient broth culture of *Escherichia coli*.
  2. 24-hr. nutrient broth culture of *Escherichia communior*.
  3. 24-hr. nutrient broth culture of *Staphylococcus aureus*.
  4. 24-hr. nutrient broth culture of *Proteus vulgaris*.
  5. 4 tubes of glucose fermentation broth with bromothymol blue indicator.
  6. 4 tubes of lactose fermentation broth with bromothymol blue indicator.
  7. 4 tubes of sucrose fermentation broth with bromothymol blue indicator.
  8. 4 tubes of mannose fermentation broth with bromothymol blue indicator.
  9. 4 tubes of dextrin fermentation broth with bromothymol blue indicator.

Procedure:

  1. Inoculate one set of the fermentation tubes each with one loopful of a 24-hr. nutrient broth culture of *E. coli*.
  2. Inoculate a second set of tubes each with one loopful of a 24-hr. nutrient broth culture of *E. communior*.
  3. Inoculate a third set of tubes each with one loopful of a 24-hr. nutrient broth culture of *S. aureus*.
  4. Inoculate the last set of tubes each with one loopful of a 24-hr. nutrient broth culture of *P. vulgaris*.
  5. Incubate all tubes at 37°C. for 48 hr.
  6. Record your results in the following table:

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Organism	Reaction				
	Glucose	Lactose	Sucrose	Mannose	Dextrin
E. coli					
E. communior					
S. aureus					
P. vulgaris					

Record your results as follows: A = acid; AG = acid + gas; O = alkaline

Questions:

1. Can you identify the foregoing organisms on the basis of their fermentation reactions?

2. Why is bromothymol blue, when added to a neutral medium, superior to bromocresol purple for measuring acid and alkaline production?

3. Are fermentations aerobic or anaerobic? Why?

4. If you inoculated a tube of nutrient broth and a tube of glucose broth each with one loopful of a culture of *E. coli*, which tube would show the higher cell count at the end of 24 hr.? Why?

5. Why do organisms attack carbohydrates?

6. What is meant by the statement, "The final hydrogen-ion concentration of *E. coli* in glucose broth is a physiological constant"?

References

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1942.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 49

LACTOSE BROMOCRESOL PURPLE AGAR MEDIUM

The ability of an organism to produce a fermentation can be determined very easily by streaking a loopful of a culture over the surface of nutrient agar containing the carbohydrate and a suitable indicator. Fermentation of the compound results in an increase in the hydrogen-ion concentration of the medium. The visible result is a change in the color of the agar.

If the colonies on the plate are well isolated, the change in the color of the agar remains confined to areas immediately surrounding the colonies. If the culture consists of a mixture of two bacterial species, only one of which is capable of fermenting the carbohydrate, separation of the two organisms may be accomplished. This is only possible provided the culture is first highly diluted and, also, provided the two organisms are present in approximately equal numbers.

The method is valuable for the separation and identification of species.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli.*
2. 24-hr. nutrient broth culture of *Alcaligenes faecalis.*
3. 4 tubes of lactose bromocresol purple agar.
4. 4 sterile Petri dishes.
5. 1 sterile 99-cc. water blank.
6. Sterile 1-cc. pipettes.

Procedure:

1. Melt four tubes of lactose bromocresol purple agar in an Arnold sterilizer or in a pan of boiling water.
2. Allow the agar to cool to a temperature of about 50°C.
3. Pour the melted and cooled agar into four sterile Petri dishes.
4. Set the plates aside until the agar is firm.
5. Immerse the wire loop in a culture of *E. coli.* Shake off as much of the culture as possible by striking the loop against the sides of the tube. Remove the loop from the tube and streak over the surface of one of the plates.
6. Do likewise with the culture of *A. faecalis.*
7. Pipette 0.5 cc. of the culture of *E. coli* and 0.5 cc. of the culture of *A. faecalis* into a sterile 99-cc. water blank. Use a fresh pipette for each culture.
8. Mix thoroughly This gives a dilution of about 1:200 for each culture.
9. Remove one loopful of the mixed suspension and streak over the surface of a third plate.
10. Without reinoculating the loop, streak it over the surface of the remaining plate.
11. Invert all plates and incubate at 37°C. for 24-hr.
12. After you have examined the plates, keep them in your locker for 2 weeks and observe again.
13. Record all results in the following table:

Organism	Color of agar	
	24 hr.	Two weeks
E. coli.		
A. faecalis		

Questions:

1. Why is bromocresol purple more satisfactory than phenol red for indicating changes to the acid side of neutrality?

2. Under what conditions would phenol red be more useful?

3. Approximately what pH does *E. coli* produce in the above medium?

4. What results would you expect if the above medium were heavily buffered with phosphate to give an initial pH of 7.0 and inoculated with *E. coli?*

5. Why do the plates, after being stored for two weeks, change in color from yellow back to purple?

References

Committee on Bacteriological Technic: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1942.

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 50

LACTOSE LITMUS AGAR MEDIUM

Litmus is still much used as an indicator in bacteriological media but it does not give accurate results in terms of hydrogen-ion concentration. It is not a delicate detector of changes in acidity and alkalinity. For this reason it is being slowly replaced by the more sensitive sulfonephthalein indicators.

Litmus possesses one advantage over the sulfonephthalein indicators in that it is sensitive to decolorization by bacteria. It indicates changes in the oxidation-reduction potential of the system. The dye is colored when oxidized and colorless when reduced. The decolorization of the indicator is first noted in the deeper layers of the culture media.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli.*

2. 24-hr. nutrient broth culture of *Bacillus subtilis.*

3. 24-hr. nutrient broth culture of *Staphylococcus aureus.*

4. 3 tubes of lactose litmus agar medium.

Procedure:

1. Melt three tubes of lactose litmus agar in an Arnold sterilizer or in a pan of boiling water.

2. Allow the agar to cool to a temperature of about 45°C.

3. Inoculate a tube with one loopful of a 24-hr. nutrient broth culture of *E. coli.*

4. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *B. subtilis.*

5. Inoculate the last tube with one loopful of a 24-hr. nutrient broth culture of *S. aureus.*

6. Mix thoroughly by rolling the tubes between the palms of the hands.

7. Incubate the tubes at 37°C. for 48 hr.

8. Record your results in the following table:

Organism	Reaction	Gas	Decolorization of litmus
E. coli			
B. subtilis			
S. aureus			

Questions:

1. What is litmus?
2. What is azolitmin?
3. Why do organisms decolorize the indicator?
4. Is the decolorization an aerobic or an anaerobic reaction?
5. How can the color be restored?

References

COMMITTEE ON BACTERIOLOGICAL TECHNIC: "Manual of Methods for Pure Culture Study of Bacteria," Geneva, N. Y., Society of American Bacteriologists, 1942.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 51

THE ACETIC ACID BACTERIA

Vinegar is the product resulting from the oxidation of alcohol to acetic acid by bacteria.

When a dilute alcoholic liquid is exposed to the air, a film slowly develops on its surface. At the same time the liquid becomes sour, owing to the oxidation of the alcohol to acetic acid. The film is composed of a viscous, gelatinous material, or zooglea, in which are embedded the acetic acid bacteria. This film is sometimes referred to as mother of vinegar because a small portion acts as a starter when added to more alcoholic liquid.

The acetic acid bacteria belong to the genus *Acetobacter*. They oxidize alcohol to acetic acid in the presence of a plentiful supply of air. That is why their growth is usually confined to the surface of alcoholic liquids.

Required:

1. Sample of vinegar containing a film of mother of vinegar.
2. Slides and cover slips.

Procedure:

1. Remove some of the zoogleal material from a sample of vinegar and smear out on a glass slide.

2. Stain by Gram's method and examine the slide under the oil-immersion objective.

3. Test for motility by the hanging-drop method.

4. Record your results in the following table:

Gram reaction	Motility	Morphology

Questions:

1. What can you say about the motility of the various species of acetic acid bacteria?

2. What is the optimum reaction for the growth of the various species?

3. Are the organisms capable of fermenting glucose to acetic acid?

4. Write the reaction for the conversion of alcohol to acetic acid.

5. Do the organisms exhibit pleomorphism?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE DISSOCIATION OF BACTERIA

EXERCISE 52

THE EFFECT OF CHEMICALS ON DISSOCIATION

A culture of *Escherichia coli*, streaked over the surface of agar containing a small amount of sodium monochloroacetate, dissociates into two types of colonies: (1) a normal type and (2) a variant type that fails to produce gas from glucose, levulose, mannose, galactose, arabinose, xylose, lactose, dextrin, and salicin, but is still capable of producing gas from dulcitol, mannitol, and sorbitol. The variant retains the new characteristics for several generations. In other respects the organisms appear to be identical with those present in the original culture.

Required:

    1. 24-hr. nutrient broth culture of *Escherichia coli*.
    2. 2 tubes of sodium monochloroacetate agar.
    3. 6 tubes of glucose fermentation broth with bromothymol blue indicator.
    4. 2 sterile Petri dishes.

Procedure:

    1. Melt two tubes of sodium monochloroacetate agar in an Arnold sterilizer or in a pan of boiling water.
    2. Allow the agar to cool to a temperature of about 50°C.
    3. Pour the melted and cooled agar into two sterile Petri dishes and set aside until the agar is firm.
    4. Streak one loopful of a 24-hr. nutrient broth culture of *E. coli* over the surface of one of the plates.
    5. Without reinoculating the loop, streak it over the surface of the other plate.
    6. Inoculate a tube of glucose fermentation broth containing bromothymol blue indicator with one loopful of the same culture of *E. coli*. This serves as the control.
    7. Incubate the tube and the inverted plates at 37°C. for 4 days.
    8. Select five of the largest isolated colonies of *E. coli* appearing on the plates and transfer to each of five tubes of glucose fermentation broth containing bromothymol blue indicator.
    9. Incubate the tubes at 37°C. for 48 hr.
    10. Observe the tubes for the presence of acid and gas.
    11. Record your results in the following table:

Culture of *E. coli*	Medium	Percentage of gas in the inverted vial, approx.
Control	Glucose fermentation broth	
Colony 1	Glucose fermentation broth	
Colony 2	Glucose fermentation broth	
Colony 3	Glucose fermentation broth	
Colony 4	Glucose fermentation broth	
Colony 5	Glucose fermentation broth	

12. At least one of the tubes should fail to show gas.

13. If you did not obtain such a result compare your tubes with those of the other members of the class.

Questions:

1. Is the induced variant permanent or of short duration?

2. Name several other chemicals that have been used to induce dissociation.

3. What organism is often diagnosed by the striking involution forms produced on sodium chloride agar?

4. What may occur when an organism is repeatedly transferred to a carbohydrate medium which it normally fails to ferment?   Explain.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

ASSOCIATIONS OF BACTERIA

Exercise 53

BACTERIAL COMMENSALISM

Commensalism may be defined as the living together of two different organisms one of which is benefited by the association while the other is apparently neither benefited nor harmed. . The phenomenon is probably of frequent occurrence in nature.

Bacillus cereus develops very rapidly in a synthetic medium containing casein as the only source of nitrogen. The casein is hydrolyzed with the liberation of amino acids and other products. On the other hand, *Pseudomonas fluorescens* is unable to attack the casein but can utilize amino acids with ease.

When both organisms are inoculated into a synthetic medium containing casein as the only source of nitrogen, *B. cereus* attacks the protein molecule with the liberation of amino acids and other compounds. *P. fluorescens* then decomposes the amino acids as they are formed. The number of cells of *P. fluorescens* soon exceeds that of *B. cereus*. *P. fluorescens* is benefited by the association while *B. cereus* is probably neither benefited nor injured.

Required:

1. 24-hr. nutrient broth culture of *Pseudomonas fluorescens*.
2. 24-hr. nutrient broth culture of *Bacillus cereus*.
3. 3 tubes of Waksman and Lomanitz's synthetic medium containing 1 per cent casein.
4. Slides.

Procedure:

1. Inoculate a tube of Waksman and Lomanitz's synthetic medium, containing 1 per cent casein, with one loopful of a 24-hr. nutrient broth culture of *B. cereus*.
2. Inoculate a second tube of the medium with one loopful of a 24-hr. nutrient broth culture of *P. fluorescens*.
3. Inoculate the last tube of the medium with one loopful each of *B. cereus* and *P. fluorescens*.
4. Incubate the tubes for 48 hr. at 37°C.
5. Examine the tubes for turbidity and odor.
6. Prepare Gram stains from each tube and examine under the oil-immersion objective.
7. Note the relative numbers of the two species of cells present in the three cultures.
8. Record your results in the following table:

Organism	Relative numbers	Odor	Gram reaction	Ratio: *B. cereus/ P. fluorescens*
B. cereus				
P. fluorescens				
B. cereus, P. fluorescens				

Questions:

1. Why does the decomposition of the casein by *B. cereus* take place more rapidly in the absence of a fermentable carbohydrate?

2. What is meant by the term proteolytic?

3. Where does the phenomenon of commensalism commonly occur in Nature?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 54

BACTERIAL ANTIBIOSIS OR ANTAGONISM

Antibiosis may be defined as the living together of two species of organisms one of which is distinctly injurious to the growth of the other, resulting finally in its death.

Pseudomonas aeruginosa and *Streptococcus faecalis* form an antagonistic pair of organisms. If a loopful of a culture of each organism is inoculated into a tube of nutrient broth medium and incubated at 37°C., *P. aeruginosa* will grow at a more rapid rate than *S. faecalis*, resulting finally in the death of the latter. This occurs regardless of which organism predominates at the start.

Escherichia coli and *Bacillus aerosporus* also form an antagonistic pair. Regardless of the proportions of the two organisms present at the start, *E. coli* will always gain the ascendency and finally destroy the other member.

Required:

1. 24-hr. nutrient broth culture of *Pseudomonas aeruginosa*.
2. 24-hr. nutrient broth culture of *Streptococcus faecalis*.
3. 24-hr. nutrient broth culture of *Escherichia coli*.
4. 24-hr. nutrient broth culture of *Bacillus aerosporus*.

5. 2 tubes of nutrient broth.

6. Slides.

Procedure :

1. Inoculate a tube of nutrient broth with one loopful each of cultures of *P aeruginosa* and *S. faecalis.*

2. Inoculate a second tube of nutrient broth with one loopful each of cultures of *E. coli* and *B. aerosporus.*

3. Incubate the tubes at 37°C.

4. Prepare Gram stains from the cultures at the end of 24 and 48 hr. of incubation.

5. Examine the slides under the oil-immersion objective.

6. Record your observations in the following table:

Antagonistic pairs		Predominant organism in culture		Ratio: A/B	
		24 hr.	48 hr.	24 hr.	48 hr.
P. aeruginosa	A				
S. faecalis	B				
E. coli	A				
B. aerosporus	B				

Questions:

1. Why do some organisms antagonize others?

2. Could you demonstrate an antagonistic effect by means of culture filtrates? Explain.

3. Does this phenomenon have any practical application?   Explain.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 55

BACTERIAL SYNERGISM

Synergism may be defined as the living together of two species of organisms with the formation of gas in a carbohydrate medium which is not produced by either organism when grown separately.

Staphylococcus aureus and *Proteus vulgaris* growing in pure cultures are unable to produce gas from lactose. When the two organisms are growing in association, however, gas is produced from lactose.

S. aureus and *Escherichia coli* may be taken as another synergic pair. The two organisms cultivated in pure cultures are unable to produce gas from sucrose but are able to do so when grown in association.

Required:

1. 24-hr. nutrient broth culture of *Staphylococcus aureus*.
2. 24-hr. nutrient broth culture of *Proteus vulgaris*.
3. 24-hr. nutrient broth culture of *Escherichia coli*.
4. 3 tubes of lactose fermentation broth containing bromothymol blue indicator.
5. 3 tubes of sucrose fermentation broth containing bromothymol blue indicator.

Procedure:

1. Inoculate a tube of lactose fermentation broth with one loopful of a 24-hr. culture of *S. aureus*.

2. Inoculate a second tube of lactose fermentation broth with one loopful of a 24-hr. culture of *P. vulgaris*.

3. Inoculate a third tube of lactose fermentation broth with one loopful each of a 24-hr. culture of *S. aureus* and of *P. vulgaris*.

4. Label the tubes and set aside.

5. Inoculate a tube of sucrose fermentation broth with one loopful of a 24-hr. culture of *S. aureus*.

6. Inoculate a second tube of sucrose fermentation broth with one loopful of a 24-hr. culture of *E. coli*.

7. Inoculate a third tube of sucrose fermentation broth with one loopful each of a 24-hr. culture of *S. aureus* and of *E. coli*.

8. Label the tubes.

9. Incubate both sets at 37°C. for 48 hr.

10. Record your observations in the following table:

Organism	Lactose broth		Organism	Sucrose broth	
	Acid	Gas		Acid	Gas
S. aureus			*S. aureus*		
P. vulgaris			*E. coli*		
S. aureus and *P. vulgaris*			*S. aureus* and *E. coli*		

Questions:

1. Discuss briefly the mechanism of bacterial synergism.

2. Where would you expect this phenomenon to be of common occurrence?

3. In conducting bacteriological examinations of water for the presence of coliform organisms, how could false-positive presumptive tests due to bacterial synergism be largely eliminated?

4. How does synergism differ from commensalism?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIOLOGY OF AIR

EXERCISE 56

THE ORGANISMS IN AIR

Air is not a natural environment for the growth and reproduction of microorganisms. Nevertheless organisms are found in air and their presence is of considerable importance economically and to public health.

Organisms are introduced into air chiefly by means of dust particles that contain dry vegetative cells and spores. The organisms are for the most part saprophytes, which are the forms responsible for contaminations from the air.

This experiment demonstrates that many kinds of organisms are found in air and that their presence may be responsible for the contamination of culture media. Therefore, it is of utmost importance that aseptic precautions be observed to prevent entrance of outside organisms into laboratory cultures.

Required:

1. 3 tubes of nutrient agar.
2. 3 sterile Petri dishes.
3. Slides.

Procedure:

1. Melt three tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
2. Allow the agar to cool to a temperature of about 50°C.
3. Pour each tube of agar into a sterile Petri dish and set aside until firm.
4. Expose one dish on the laboratory table for 20 min.
5. Expose the other dish in a quiet room for the same period of time.
6. The third dish serves as a control.
7. At the end of 20 min. replace the lids, invert the plates, and incubate at 37°C. for 48 to 72 hr.
8. Note the types of colonies appearing on the plates.
9. Prepare Gram stains from the various kinds of colonies.
10. Record your results in the following table:

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EXAMINATION OF COLONIES APPEARING ON AIR PLATES

Locality	Number of colonies	Macroscopic appearance of colonies	Morphology of organisms	Gram reaction	Pigmented colonies	Mold colonies
Laboratory						
Quiet room						

Questions:

1. Are pigmented organisms found in the air? Why?
2. Are molds and yeasts found in the air? Why?
3. Why is *Bacillus subtilis* universally present in the atmosphere?
4. Did you notice anything unusual about the Gram reaction of the coccus forms?
5. Is this a quantitative method for the enumeration of the air population?
6. How could you make a quantitative examination of air?
7. What is meant by air-borne infection?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIOLOGY OF WATER

Exercise 57

THE QUANTITATIVE EXAMINATION OF WATER

The methods generally employed in the laboratory for the quantitative bacteriological examination of water give only a fraction of the total count. The majority of organisms found in water fail to grow on the usual laboratory media. However the sanitary bacteriologist is not interested in the enumeration of all organisms but in a group of rich-food-loving organisms found in sewage.

The number of bacteria present in a water sample, capable of developing on a standard agar medium, should not exceed 300 per cubic centimeter. If a sample is suspected of containing a higher count, appropriate dilutions should be prepared.

Required:

1. Sample of water.
2. 6 tubes of nutrient agar.
3. Two 9-cc. water blanks.
4. Sterile 1-cc. pipettes.
5. 6 sterile Petri dishes.

Procedure:

1. Label six sterile Petri dishes as follows: two 1 cc.; two 0.1 cc; and two 0.01 cc.
2. Shake the water sample at least 25 times to obtain a uniform distribution of organisms.
3. By means of a sterile 1-cc. pipette, transfer 1 cc. of the water sample to each of two Petri dishes.
4. Using the same pipette, transfer 1 cc. of the water sample to a 9-cc. water blank.
5. Mix thoroughly by rotating the tube between the palms of the hands.
6. Transfer 1 cc. of the 1:10 dilution to each of two Petri dishes. Use a fresh pipette.
7. Using the same pipette and dilution, transfer 1 cc. to another 9-cc. water blank.
8. Mix thoroughly as before.
9. Transfer 1 cc. of the 1:100 dilution to each of two Petri dishes. Use a fresh pipette.
10. Melt six tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
11. Allow the agar to cool to a temperature of about 50°C.
12. Pour the melted and cooled agar into the Petri dishes and tilt from side to side to obtain a uniform distribution of organisms.
13. When the agar has hardened, invert the plates and incubate at 37°C. for 24 hr.

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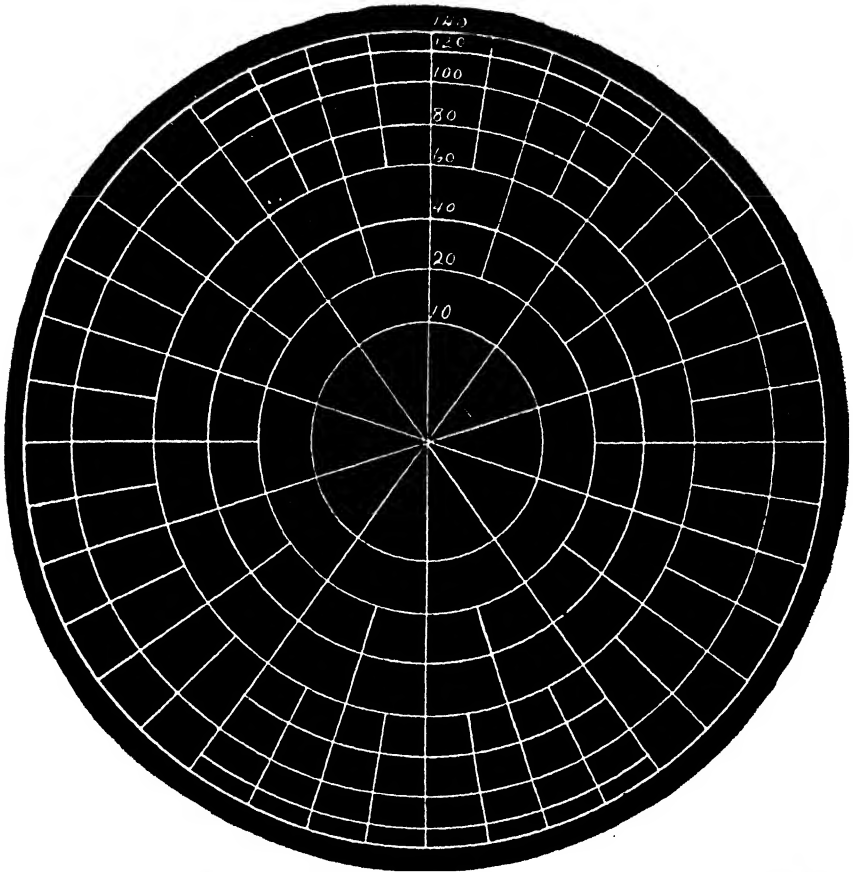
FIG. 4.—Counting plate. Tear out along perforations and use for estimating the number of colonies developing on agar plates. It is better to paste it on a piece of heavy cardboard. (*From Heinemann, "Laboratory Guide in Bacteriology," University of Chicago Press.*)

14. Discard the plates showing more than 300 colonies.
15. Count the colonies developing on the remainder of the dishes.
16. Calculate the number of organisms per cubic centimeter of water sample.

Questions:

1. Does the quantitative examination of water have any sanitary significance?
2. Why do not all organisms present in water develop on agar plates?
3. Why do some organisms multiply in water whereas others do not?
4. Why do disease organisms quickly die when introduced into a water supply?

References

American Public Health Association: "Standard Methods for the Examination of Water and Sewage," New York, 1936.

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 58

THE PRESUMPTIVE TEST

Tests for the presence of members of the colon group are based on the fact that such organisms are capable of fermenting lactose with the production of acid and gas. The formation of 10 per cent or more of gas in 24 hr. at 37°C. constitutes presumptive evidence for the presence of members of the colon group. The presence of gas in any amount after 48 hr. of incubation constitutes a doubtful presumptive test and requires further examination. The absence of gas after 48 hr. constitutes a negative presumptive test and further tests are not required.

Required:

1. Sample of water.
2. 5 tubes of lactose fermentation broth each containing 20 cc. of medium and bromothymol blue indicator.
3. 1 tube of lactose fermentation broth containing 5 cc. of medium and bromothymol blue indicator.
4. Sterile 1-cc. pipette.
5. Sterile 10-cc. pipette.

Procedure:

1. Inoculate five tubes of lactose fermentation broth, containing 20 cc. of medium, each with 10 cc. of the water sample.
2. Inoculate one tube of lactose fermentation broth, containing 5 cc. of medium, with 1 cc. of the water sample.
3. Incubate all tubes at 37°C. for 48 hr.
4. Examine the tubes for the presence of acid and gas at the end of 24 and 48 hr. of incubation. If acid is produced and the inverted vials show the presence of at least 10 per cent of gas, the presumptive test is positive. If no gas or less than 10 per cent appears in the inverted vials in 24 hr., reincubate the tubes for another 24 hr.
5. The presence of gas in any amount after 24 hr. constitutes a doubtful presumptive test. The absence of gas after an incubation period of 48 hr. constitutes a negative test.

6. If the presumptive test is negative, the tubes are discarded and no further tests are required. If the presumptive test is positive or doubtful, the tubes are retained for Exercises 59 and 60.

7. Record all results in the following table:

Fermentation tube	Acid	Gas	Gas, %	Presumptive test		
				Positive	Negative	Doubtful
10 cc. water						
10 cc. water						
10 cc. water						
10 cc. water						
10 cc. water						
1 cc. water						

Questions:

1. Why is *Escherichia coli* referred to as an indicator organism in water analysis?
2. What can be said concerning the viability of *E. coli* and *E. typhosa* in a water sample?
3. Why is lactose used in preference to glucose in the presumptive test?
4. What is meant by bacterial synergism?
5. Does the phenomenon play an important role in the presumptive test?
6. How can synergism be largely eliminated in the presumptive test?
7. What advantage does a direct plating examination possess over a preliminary enrichment procedure? What disadvantage?

References

AMERICAN PUBLIC HEALTH ASSOCIATION: "Standard Methods for the Examination of Water and Sewage," New York, 1936.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

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EXERCISE 59

EOSIN METHYLENE BLUE AGAR MEDIUM

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Eosin methylene blue agar is prepared by dissolving an appropriate amount of lactose and the two dyes, eosin and methylene blue, in a nutrient agar base and the mixture poured into Petri dishes.

Typical colonies of *Escherichia coli* developing on this medium show the presence of dark centers and a greenish metallic sheen. On the other hand, typical colonies of *Aerobacter aerogenes* show brown centers without a metallic sheen.

The medium is of great value for the separation of the *Escherichia* and *Aerobacter* divisions of the colon group.

Required:

1 Eosin methylene blue (E.M.B.) agar plate.
2. Lactose fermentation tube showing a positive presumptive test.
3. 24-hr. nutrient broth culture of *Escherichia coli.*
4. 24-hr. nutrient broth culture of *Aerobacter aerogenes.*

Procedure:

1. Invert the plate and divide into three sectors by means of a china marking pencil.

2. Streak on one sector a loopful of a 24-hr. nutrient broth culture of *E. coli.*

3. Streak on another sector a loopful of a 24-hr. nutrient broth culture of *A. aerogenes.*

4. Streak on the remaining sector a loopful of culture from the lactose fermentation tube showing a positive presumptive test.

5. Mark each sector with the name of the culture used.

6. Invert the plate and incubate at 37°C. for 24 hr.

7. Examine the plate for the presence of typical colonies possessing a brilliant metallic sheen. The presence of such colonies after an incubation period of 24 hr. constitutes a positive confirmed test.

8. If no typical colonies have developed within 24 hr., the test cannot be considered negative since it sometimes happens that members of the *Escherichia* group fail to form such colonies on E.M.B. agar, or that the colonies develop slowly.

9. Under these conditions reincubate the plate for another 24 hr. and retain for Exercise 61.

Questions:

1. How is E.M.B. agar prepared?
2. Why should the prepared plates not be exposed to direct sunlight?
3. Is the medium stable?
4. Why do typical colonies of *E. coli* have dark centers?
5. What is the purpose of the eosin?
6. Will *Bacillus subtilis* grow when steaked over the surface of an E.M.B. agar plate? Explain.
7. Why is it customary to distinguish between the *Escherichia* and *Aerobacter* divisions of the colon group?

References

AMERICAN PUBLIC HEALTH ASSOCIATION: "Standard Methods for the Examination of Water and Sewage," New York, 1936.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 60

ENDO AGAR MEDIUM

Endo medium is prepared by adding basic fuchsin, previously decolorized by sodium sulfite, and lactose to a melted nutrient agar base and the mixture poured into Petri dishes.

Typical colonies of *Escherichia coli* developing on this medium possess a gold-like metallic sheen. At the same time the red color of the fuchsin is restored. It is believed that Endo agar acts as a trapping medium for the acetaldehyde, which is the compound responsible for the characteristic reaction. Acetaldehyde is an intermediate product in the fermentation of lactose. The acetaldehyde forms an addition product with the sodium sulfite. The dye-sulfite combination is then broken, resulting in the restoration of the red color. The metallic sheen imparted to the colonies is due to the precipitation of the liberated dye by the organic acids produced from the lactose. On the other hand, typical colonies of *Aerobacter aerogenes* do not possess a metallic sheen and the red color is not restored.

The medium is of value for the separation of the *Escherichia* and *Aerobacter* divisions of the colon group.

Required:

1. 1 Endo agar plate.
2. Lactose fermentation tube showing a positive presumptive test.
3. 24-hr. nutrient broth culture of *Escherichia coli*.
4. 24-hr. nutrient broth culture of *Aerobacter aerogenes*.

Procedure:

1. Invert the plate and divide into three sectors by means of a china marking pencil.
2. Streak on one sector a loopful of a 24-hr. nutrient broth culture of *E. coli*.
3. Streak on another sector a loopful of a 24-hr. nutrient broth culture of *A. aerogenes*.
4. Streak on the remaining sector a loopful of culture from the lactose fermentation tube showing a positive presumptive test.
5. Mark each sector with the name of the culture used.
6. Invert the plate and incubate at 37°C. for 24 hr.
7. Examine the plate for the presence of typical colonies of *E. coli*. The red color of the fuchsin should be restored and the colonies should possess a brilliant metallic sheen. The presence of typical colonies on Endo agar after an incubation period of 24 hr. constitutes a positive confirmed test.

8. If no typical colonies have developed within 24 hr., the test cannot be considered negative since it sometimes happens that members of the *Escherichia* fail to form such colonies on Endo agar, or that the colonies develop slowly.

9. Under these conditions reincubate the plate for another 24 hr. and retain for Exercise 61.

Questions:

1. How is Endo agar prepared?
2. Is Endo agar stable?
3. What happens when sterile Endo agar plates are exposed to sunlight?
4. Why is lactose added to the medium?
5. Discuss the mechanism responsible for the characteristic reaction.
6. Could crystal violet be used instead of basic fuchsin? Explain.

References

AMERICAN PUBLIC HEALTH ASSOCIATION: "Standard Methods for the Examination of Water and Sewage," New York, 1936.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 61

THE COMPLETED TEST

The purpose of the completed test is to determine if the organism isolated from the presumptive broth culture is again capable of fermenting lactose with the production of acid and gas; also if the agar slant culture shows the presence of small, aerobic nonspore-forming, Gram-negative rods. If these conditions are fulfilled, the organism is considered to be a member of the colon group.

Required:

1. Endo or E.M.B. agar plate on which appear colonies from a positive presumptive test.
2. 1 lactose fermentation tube containing bromothymol blue indicator.
3. 1 nutrient agar slant.

Procedure:

1. Remove a portion of a typical colony appearing on an Endo or an E.M.B. agar plate and transfer to a lactose broth fermentation tube.

2. Remove another portion of the same colony and streak over the surface of a nutrient agar slant.

3. If no typical colonies appear on the plate, after incubation periods of 24 and 48 hr., transfer a portion of a representative colony to a lactose broth fermentation tube, and another portion to the surface of a nutrient agar slant.

4. It is best to transfer two colonies to two fermentation tubes and to two agar slants.

5. Incubate the lactose fermentation tubes at 37°C. for 48 hr.

6. Incubate the agar slants at 37°C. for 24 hr.

7. Examine the growth on the agar slants for the presence of small, Gram-negative, nonspore-forming rods.

8. The presence of acid and gas in lactose broth within 48 hr. and of small, non-spore-forming, Gram-negative rods on the agar slants constitutes a positive completed test for members of the colon group.

Questions:

1. Which organisms are included in the colon group?

2. Does the standard procedure attempt to separate the various members of the colon group?

3. What are fecal and nonfecal colon organisms?

4. Why is it usually customary to separate the colon group into fecal and nonfecal types?

<div align="center">Reference</div>

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

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EXERCISE 62

THE VOGES-PROSKAUER TEST

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A positive Voges-Proskauer reaction depends upon the presence of acetylmethylcarbinol, an intermediate product of carbohydrate metabolism. This compound is produced by *Aerobacter aerogenes* but not by *Escherichia coli*. The acetylmethylcarbinol in the presence of sodium hydroxide and air is further oxidized to diacetyl which, in the presence of peptone, gives an eosin-like color.

The Voges-Proskauer test appears to possess considerable sanitary significance since it distinguishes to a high degree between typical fecal and typical nonfecal members of the colon group.

Required:

1. Nutrient agar slant culture of the organism isolated under the completed test.

2. 24-hr. nutrient broth culture of *Escherichia coli*.

3. 24-hr. nutrient broth culture of *Aerobacter aerogenes*.

4. 3 tubes of methyl red broth.

5. 2 per cent aqueous solution of ferric chloride.

6. 10 per cent aqueous solution of sodium hydroxide.

Procedure:

1. Inoculate one tube of methyl red broth from the agar slant culture of the organism isolated under the completed test.

2. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *E. coli*.

3. Inoculate a third tube with one loopful of a 24-hr. nutrient broth culture of *A. aerogenes*.

4. Incubate the tubes at 37°C. for four days.

5. At the end of the incubation period add to 5 cc. of the culture 2 drops of a 2 per cent solution of ferric chloride and 5 cc. of a 10 per cent solution of sodium hydroxide. Mix well by shaking.

6. The ferric chloride must be added before the addition of the sodium hydroxide solution. If it is added after the addition of the alkali, a marked flocculation of ferric hydroxide occurs.

7. A positive test is indicated by a deep copper coloration, which appears at the surface after a few minutes and extends to the bottom of the tube. The color remains for several days.

8. The test is usually performed by omitting the ferric chloride, in which case positive cultures show a pink color. The addition of ferric chloride, however, hastens the oxidation of acetylmethylcarbinol to diacetyl, giving a positive test in a few minutes.

9. Compare the unknown culture with the cultures of *E. coli* and *A. aerogenes*.

Questions:

1. Does acetylmethylcarbinol, in the presence sodium hydroxide, produce a pink color?

2. Do mixed cultures of *A. aerogenes* and *E. coli* give positive Voges-Proskauer tests? Explain.

3. What constituent of peptone is required for a positive test?

4. What is the origin of the acetylmethylcarbinol?

5. How specific is the reaction?

References

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

WERKMAN, C. H.: An Improved Technic for the Voges-Proskauer Test, *J. Bact.*, **20:** 121, 1930.

EXERCISE 63

THE METHYL RED TEST

The methyl red test is a measure of the amount of acid produced by members of the colon group. From a given amount of carbohydrate *Escherichia coli* produces more acid than *Aerobacter aerogenes*. An amount of carbohydrate that is just sufficient for *E. coli* to produce its limiting hydrogen-ion concentration is inadequate for *A. aerogenes* to produce its maximum acidity. The result is that *E. coli* produces no further change whereas *A. aerogenes* first exhausts the carbohydrate and, being insufficient for it to produce its limiting hydrogen-ion concentration, then attacks the nitrogenous constituents of the medium for both structure and energy. Under these conditions the medium becomes progressively more alkaline.

Typical strains of *E. coli* produce sufficient acid to give a red color with methyl red indicator; typical strains of *A. aerogenes* give a yellow or orange color.

Required:

1. 24-hr. nutrient broth culture of the organism isolated under the completed test.

2. 24-hr. nutrient broth culture of *Escherichia coli*.

3. 24-hr. nutrient broth culture of *Aerobacter aerogenes*.

    4. 3 tubes of methyl red broth.

    5. Solution of methyl red indicator.

Procedure:

    1. Inoculate a tube of methyl red broth with one loopful of a 24-hr. nutrient broth culture of *E. coli.*

    2. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *A. aerogenes.*

    3. Inoculate a third tube with one loopful of a 24-hr. nutrient broth culture of the organism isolated under the completed test.

    4. Incubate the tubes at a temperature of 37°C. for 48 hr.

    5. Remove the tubes from the incubator and add 5 drops of methyl red indicator solution to each.   Mix thoroughly.

    6. Record a distinct red color as methyl red +; a distinct yellow color as methyl red −.   Intermediate colors should be recorded as questionable.

Questions:

    1. How is methyl red broth prepared?

    2. How is methyl red indicator solution prepared?

    3. Could bromothymol blue be substituted for the methyl red?   Explain.

    4. "The methyl red test is a qualitative test for acid production."   Is this statement correct?   Explain.

    5. How does a change in the buffer content of the medium influence the final hydrogen-ion concentration of the culture?

    6. What results would you expect from mixed cultures of *E. coli* and *A. aerogenes?*

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 64

THE URIC ACID TEST

    Typical strains of *Aerobacter aerogenes* are capable of utilizing uric acid as the only source of nitrogen.   Typical strains of *Escherichia coli* are unable to attack the compound.   The result is that *A. aerogenes* is able to grow and multiply in an inorganic medium containing uric acid as the only nitrogen compound whereas *E. coli* fails to develop.

    The results correlate almost 100 per cent with the Voges-Proskauer and methyl red tests.

Required:

    1. Nutrient agar slant culture of the organism isolated under the completed test.

    2. 24-hr. nutrient broth culture of *Escherichia coli.*

    3. 24-hr. nutrient broth culture of *Aerobacter aerogenes.*

    4. 3 tubes of Koser's uric acid medium.

Procedure:

    1. Inoculate a tube of Koser's uric acid medium with one loopful of a 24-hr. nutrient broth culture of *E. coli.*

2. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *A. aerogenes*.

3. Inoculate a third tube with some of the growth from an agar slant culture of the organism isolated under the completed test.

4. Incubate all tubes at 37°C. for 4 days.

5. Record growth as + or −.

Questions:

1. How is Koser's uric acid medium prepared?
2. What care should be observed in the selection of the ingredients for the medium?
3. What precautions should be taken in preparing and storing the medium?
4. Could xanthine or hypoxanthine be substituted for the uric acid? Explain.
5. What important disadvantage does the test possess?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 65

THE SODIUM CITRATE TEST

Typical strains of *Aerobacter aerogenes* are capable of utilizing sodium citrate as the only source of carbon. Typical strains of *Escherichia coli* are unable to attack the compound. The result is that *A. aerogenes* is able to grow and multiply in an inorganic medium containing sodium citrate as the only carbon compound whereas *E. coli* fails to develop.

It has been shown that citrate utilization correlates more closely with the source of the organisms than do any of the other differential tests.

Required:

1. Nutrient agar slant culture of the organism isolated under the completed test.
2. 24-hr. nutrient broth culture of *Escherichia coli*.
3. 24-hr. nutrient broth culture of *Aerobacter aerogenes*.
4. 3 tubes of Koser's citrate medium.

Procedure:

1. Inoculate a tube of Koser's citrate medium with one loopful of a 24-hr. nutrient broth culture of *E. coli*.

2. Inoculate a second tube with one loopful of a 24-hr. nutrient broth culture of *A. aerogenes*.

3. Inoculate a third tube with a small amount of the growth from the agar slant culture of the organism isolated under the completed test.

4. Incubate all tubes at 37°C. for 4 days.

5. Record growth as + or −.

Questions:

1. How is Koser's citrate medium prepared?
2. Discuss at least three disadvantages to the use of the medium.

3. What would happen if you incubated the tubes for one week instead of only 4 days?

References

AMERICAN PUBLIC HEALTH ASSOCIATION: "Standard Methods for the Examination of Water and Sewage," New York, 1936.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIOLOGY OF MILK AND MILK PRODUCTS

EXERCISE 66

THE QUANTITATIVE EXAMINATION OF MILK

Two methods are generally followed for enumerating the number of organisms in milk: (1) the agar plate method and (2) the direct microscopic method. Both methods have their advantages and disadvantages. No single method is satisfactory for enumerating the total viable population of milk. The first method is used to a greater extent than the second.

The quantitative bacteriological examination of milk possesses considerable sanitary significance. It is an index of the conditions under which the milk is collected, handled, and stored.

Required:

1. Sample of milk.
2. Three 9-cc. water blanks.
3. 4 sterile 1-cc. pipettes.
4. 6 sterile Petri dishes.
5. 6 tubes of nutrient agar.

Procedure:

1. Place six sterile Petri dishes on your desk in an inverted position.
2. Mark two plates 1:10, two 1:100, and two 1:1000.
3. Shake the sample of milk vigorously at least 25 times to obtain a uniform suspension of the organisms.
4. Remove 1 cc. of the sample with a sterile 1-cc. pipette and transfer to a 9-cc. water blank.
5. Mix thoroughly by aspirating and expelling several times with the pipette.
6. With the same pipette, transfer 1 cc. of the 1:10 dilution to each of the two marked Petri dishes and 1 cc. to a second 9-cc. water blank.
7. Mix the contents of the tube in the same manner as given under (5).
8. Transfer 1 cc. of the 1:100 dilution to each of the two marked Petri dishes and 1 cc. to a third 9-cc. water blank.
9. Again mix the contents of the tube thoroughly.
10. Transfer 1 cc. of the 1:1000 dilution to each of the two marked Petri dishes.
11. Melt six tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
12. Allow the agar to cool to a temperature of about 45°C.

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13. Pour the melted and cooled agar into the Petri dishes.

14. Mix thoroughly by tilting and rotating the dishes.

15. When the agar has solidified invert the plates and incubate at 37°C. for 48 hr.

16. Only those plates showing from 30 to 300 colonies should be counted. If there are no plates showing colonies within these limits the plate that comes the nearest to 300 should be counted. Dishes showing less than 20 colonies should not be counted unless it happens that no other plates have been prepared.

17. Record your results in the following table:

Sample	Plate	Dilutions			Count per cc.
		1:10	1:100	1:1000	
	1				
	2				

18. Calculate the bacterial count per cubic centimeter of milk from the dilution showing the required number of colonies on the plates.

Questions:

1. Did your duplicate plates check closely?

2. Why should the sample be well shaken before withdrawing a measured amount of milk?

3. Why do not all organisms present in milk grow when embedded in nutrient agar?

4. Name the important factors that influence plate counts of milk.

5. What effect would an incubation temperature of 35°C., rather than 37°C., have on milk counts? Explain.

6. Discuss some important objections to the plate-count method.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 67

THE REDUCTASE TEST

Methylene blue is of value in making a rapid survey of the quality of milk. The dye is decolorized by bacteria, the rate being dependent upon the number of organisms' present (see page 72). The test can be employed to determine, in a rough way, the bacterial count of the milk

sample. The procedure is quickly and easily carried out and with a minimum of expense. It is particularly valuable in making a rapid inspection of a large number of samples to determine if the milk answers the requirements prescribed by law.

Required:

1. Sample of grade *A* milk.
2. Sample of grade *B* milk.
3. Sample of raw milk.
4. 1:20,000 aqueous solution of methylene blue.
5. 3 sterile 10-cc. pipettes.
6. Sterile 1-cc. pipette.
7. 3 sterile test tubes.

Procedure:

1. Pipette 9-cc. quantities of the three grades of milk, into sterile test tubes. Use a fresh pipette for each sample.
2. Add to each tube 1 cc. of a 1:20,000 aqueous solution of methylene blue. This gives a final dye concentration of 1:200,000.
3. Cork the tubes and shake well.
4. Place the tubes in a 37°C. water bath and allow to remain for 5 min.
5. Remove the tubes from the water bath and place in the 37°C. incubator.
6. Observe the tubes at ½-hr. intervals for the first 2 hr.
7. After that make observations at the end of each hour until decolorization is complete in all tubes.
8. Record your results in the following table:

Sample	Time of decolori-zation, hr.	Range of bacterial count*	Quality of milk
A			
B			
C			

* 1. Good milk, not decolorized in 5½ hr., containing as a rule less than ½ million organisms per cubic centimeter.

2. Fair milk, decolorized in less than 5½ hr. but not less than 2 hr., containing as a rule ½ to 4 million organisms per cubic centimeter.

3. Bad milk, decolorized in less than 2 hr. but not less than 20 min., containing as a rule 4 to 20 million bacteria per cubic centimeter.

4. Very bad milk, decolorized in 20 min. or less, containing as a rule over 20 million organisms per cubic centimeter.

Questions:

   1. Why is this called the reductase test?

   2. Is the decolorization of methylene blue an intracellular or an extracellular reaction?

   3. Could other dyes be used instead of methylene blue?

   4. Why does decolorization start first at the bottom of the tube?

   5. Why is the reductase test not always in good agreement with the agar plate method?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 68

THE INFLUENCE OF TEMPERATURE UPON THE KEEPING QUALITY OF MILK

   The number of organisms present in milk at the outset depends upon the degree of care exercised in its collection and handling. After that the number increases rapidly unless the milk is stored at a low temperature. The temperature at which it is stored determines to a large extent the bacterial count and the microflora of milk.

Required:

   1. Sample of fresh milk.

   2. 4 sterile test tubes.

   3. Three 99-cc. water blanks.

   4. Twelve 9-cc. water blanks.

   5. 4 sterile 1-cc. pipettes.

   6. Sterile 10-cc. pipette.

   7. 12 tubes of nutrient agar.

   8. 12 sterile Petri dishes.

Procedure:

   1. Pipette 10 cc. of milk into each of four sterile test tubes.

   2. Place tube 1 in the incubator.

   3. Place tube 2 in your locker.

   4. Place tube 3 in the refrigerator.

   5. Tube 4 is the control and must be plated immediately.

   6. Follow the same procedure as given in Exercise 66 but prepare only one agar plate of each dilution.

   7. Incubate the plates at 37°C. for 48 hr.

   8. At the end of 48 hr., prepare 1:1000, 1:10,000 and 1:100,000 dilutions of tubes 1, 2, and 3 by the same procedure as followed under (6).

   9. All counts must be made at the end of 48 hr.

   10. Record your results in the following table:

Dilution	Counts per cc.			
	Tube 1	Tube 2	Tube 3	Control
1:10				
1:100				
1:1000				
1:10,000				
1:100,000				

11. Calculate the bacterial count per cubic centimeter of milk from the dilutions showing the required number of colonies on the plates (see step 16, Exercise 66).

Questions:

1. Interpret your results.
2. Why do bacteria multiply rapidly in milk?
3. Why does the bacterial count of raw milk decrease during the first few hours after collection?
4. How does the flora vary with different temperatures?
5. Do bacterial counts of milk possess greater significance than those of water?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 69

THE NORMAL SOURING OF MILK

The organism responsible for the normal souring of milk is *Streptococcus lactis*. Several varieties of the organism have been isolated which exhibit differences in the flavor imparted to milk, the character of the fermented milk, the rate of acid formation, the rate of litmus reduction, and in other ways.

S. lactis is not a normal inhabitant of the udders of cows. The organism has been repeatedly isolated from plants, which would suggest that this is the natural habitat of the species. The organism can be obtained from the coat of the cow. Since it is normally present in cow dung, it is believed that this is the agent responsible for the contamination of milk.

Required:

1. Sample of fresh raw milk.
2. 6 tubes of lactose bromocresol purple agar.
3. 6 sterile Petri dishes.
4. Lactose fermentation broth containing bromothymol blue indicator.
5. Sucrose fermentation broth containing bromothymol blue indicator.
6. Glucose fermentation broth containing bromothymol blue indicator.
7. Slides.

Procedure:

1. Incubate the sample of raw milk at a temperature of 25°C. for 48 hr.
2. Melt two tubes of lactose bromocresol purple agar in an Arnold sterilizer or in a pan of boiling water.
3. Allow the agar to cool to a temperature of about 50°C.
4. Pour the melted and cooled agar into two sterile Petri dishes.
5. Set the plates aside for the agar to become firm.
6. Remove a loopful of the milk sample from the tube and streak it over the surface of one of the plates. Without reinoculating the loop, streak it over the surface of the other plate.
7. Invert the plates and incubate at 37°C. for 48 hr.
8. Return the sample of milk to the 25°C. incubator and allow to remain for an additional 48 hr.
9. At the end of the second incubation period streak two more lactose bromocresol purple agar plates.
10. Invert the plates and incubate at 37°C. for 48 hr.
11. Again return the milk sample to the 25°C. incubator and allow to remain for 3 more days.
12. In the meantime make Gram stains from all of the different kinds of colonies developing on the first and second series of plates. Use only well-isolated colonies.
13. Inoculate glucose, lactose, and sucrose fermentation tubes from the different colonies developing on the plates.
14. At the end of the third and final incubation period streak the two remaining plates.
15. Invert the plates and incubate at 37°C. for 48 hr.
16. Make Gram stains from well-isolated colonies as before.
17. Note in particular the presence of yeasts and molds.
18. Inoculate glucose, lactose, and sucrose fermentation tubes from the different bacterial colonies developing on the plates.
19. Record all observations in your notebook.

Questions:

1. Are all the different forms that develop on the agar plates capable of fermenting lactose?

2. Why does the flora change from day to day?

3. Why do yeasts and molds develop late in the fermentation rather than during the early stages?

4. Does S. lactis ferment lactose with the production of acid and gas?

5. If gas appears which organisms are likely to be present? Why?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 70

SLIMY OR ROPY MILK

Milk on standing occasionally becomes slimy or ropy in consistency, in which state it may be pulled out in long threads. Sometimes the change may be very slight; at other times it may be so pronounced that the milk can be drawn out into threads 3 ft. or more in length.

Several organisms are capable of producing this condition in milk. Probably the most important species is *Alcaligenes viscosus*. This organism produces its maximum ropiness at a temperature of 18 to 20°C. Ropiness is due to the formation of capsular gum by the organisms.

Another organism producing ropiness in milk is *Micrococcus cremoris-viscosi*. This organism produces an acid coagulation of milk followed by peptonization and the appearance of gum. It grows best at a temperature of about 30°C.

The slime-producing organisms find their way into milk from the water used in the stable and dairy and also from the coats of the cows.

Required:

1. Nutrient broth culture of *Alcaligenes viscosus*.

2. Nutrient broth culture of *Micrococcus cremoris-viscosi*.

3. 2 tubes of litmus milk.

4. Slides.

Procedure:

1. Inoculate a tube of litmus milk with one loopful of a nutrient broth culture of *A. viscosus*.

2. Inoculate a second tube of litmus milk with one loopful of a nutrient broth culture of *M. cremoris-viscosi*.

3. Incubate the culture of *A. viscosus* at 30°C. and the culture of *M. cremoris-viscosi* at 20°C. Allow both to incubate for 1 week.

4. At the end of the incubation period plunge your wire loop into the cultures and see if the milk can be drawn out in long threads.

5. Prepare a Gram stain and a capsule stain from each tube and note the morphology of the organisms.

6. Record your results in the following table:

Character	Organism	
	A. viscosus	*M. cremoris-viscosi*
Morphology		
Gram stain		
Capsule stain		
MILK		
Slime present		
Litmus reduction		
Reaction		
Coagulation		
Peptonization		

Questions:

    1. What is the composition of the slimy material?

    2. Why is the litmus decolorized?

    3. Are these slime-producing organisms capable of liquefying gelatin in the presence of lactose?

    4. Do the above organisms produce slime if incubated at higher temperatures?

    5. Are other organisms, besides the two species named above, capable of producing ropiness in milk?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 71
CLOSTRIDIUM PERFRINGENS IN MILK

Clostridium perfringens (*welchii*) is an anaerobic, spore-forming organism of widespread occurrence in nature. It is present in soil and water, in the intestinal tracts of man and animals, in fish, mollusks, etc. Because of its presence in the intestinal contents of cows it is frequently present in milk. The organism is generally considered the most important etiological agent of gas gangrene.

C. perfringens is a strongly saccharolytic organism, being capable of fermenting all the common sugars with the production of acid and gas. The organism produces a characteristic "stormy fermentation" in milk. The curd becomes torn to shreds by the vigorous fermentation and evolution of gas. Under these conditions the milk proteins are not attacked.

Since *C. perfringens* is found in the intestinal contents of cows its presence in milk is usually indicative of manurial contamination.

Required:
1. Sample of good milk.
2. Sample of milk polluted with a small amount of cow manure.
3. Vaspar (a mixture of equal parts of vaseline and paraffin).
4. 2 sterile test tubes.
5. Sterile 10-cc. pipette.
6. Water bath.
7. Slides.

Procedure:
1. Pipette 10 cc. of the good milk into a sterile test tube.
2. With the same pipette, transfer 10 cc. of the polluted milk to a second sterile test tube.
3. Pour melted vaspar over the surface of each tube to a depth of about ½ in.
4. Place both tubes in a water bath, previously heated to 80°C., and allow to remain at that temperature for 5 min.
5. Remove the tubes from the water bath and incubate at 37°C. for 48 hr.
6. Prepare Gram and capsule stains from the two samples of milk.
7. Record your results in the following table:

Sample	Morphology	Gram stain	Capsules	Stormy fermentation
Good milk				
Polluted milk				

Questions:

1. What is meant by stormy fermentation?
2. Is milk a good culture medium for capsule formation?
3. Why were the milk samples heated to 80°C. for 5 min.?
4. Why were the tubes covered with vaspar?
5. What is the composition of the gas?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 72

COLORED MILK

Several organisms have been isolated from milk which are capable of elaborating brilliant-colored compounds known as pigments. Since these organisms are strongly aerobic, pigment formation is observed first in the surface layer of milk.

Three typical pigment-producing organisms isolated from milk are: (1) *Pseudomonas syncyanea*, the cause of blue milk, (2) *Serratia marcescens*, the cause of red milk, and (3) *Flavobacterium synxanthum*, the organism responsible for yellow milk. Pigment formation by these organisms occurs best at a temperature of 25 to 30°C.

The organisms are found in air, soil, and water and gain entrance to milk through the use chiefly of contaminated utensils. Their presence is, therefore, largely preventable.

Required:

1. Nutrient broth culture of *Pseudomonas syncyanea*.
2. Nutrient broth culture of *Serratia marcescens*.
3. Nutrient broth culture of *Flavobacterium synxanthum*.
4. 3 tubes of sterile milk.
5. Slides.

Procedure:

1. Inoculate a tube of milk with one loopful of a nutrient broth culture of *P. syncyanea*.
2. Inoculate a second tube of milk with one loopful of a nutrient broth culture of *S. marcescens*.
3. Inoculate a third tube of milk with one loopful of a nutrient broth culture of *F. synxanthum*.
4. Incubate the tubes at 30°C. for 1 week.
5. Prepare Gram stains and examine under the oil-immersion objective.
6. Record your results in the following table:

Organism	Milk			Organisms		
	Reaction	Coagulation	Peptonization	Morphology	Gram stain	Pigment
P. syncyanea						
S. marcescens						
F. synxanthum						

Questions:

1. Are the pigments soluble in milk?

2. Does pigment formation appear first at the top or bottom? Why?

3. What can you say, in general, about the oxygen requirements of pigmented organisms?

4. What effect would higher temperatures of incubation have on pigment formation?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 73

THE PASTEURIZATION OF MILK

The destruction of all organisms in milk is called sterilization. Such milk possesses two serious objections: (1) The cooked flavor is not so pleasant as that of unheated milk and (2) heating to a high temperature may result in a decrease in the vitamin content. These objections are largely overcome by heating milk to temperatures lower than that required to sterilize but high enough to destroy all disease organisms.

The destruction of disease organisms in milk without resorting to complete sterilization is called pasteurization. Two methods are generally followed commercially: (1) the flash method and (2) the holder method. In the former method the milk is heated to a temperature of 80°C. for 2 min., then quickly cooled. The latter method makes use of a temperature of 60 to 65°C. for 30 min. In this country the holder method is used to a greater extent than the flash method.

The pasteurization process reduces the bacterial coun' from 90 to 100 per cent. This varies depending upon the number and kinds of organisms present in the milk at the time of heating.

Required:

1. Sample of raw milk.
2. Six 9-cc. water blanks.
3. 6 sterile Petri dishes.
4. 6 tubes of nutrient agar.
5. Sterile 10-cc. pipette.
6. 2 sterile 1-cc. pipettes.
7. 1 sterile test tube.
8. Water bath and thermometer.
9. Slides.

Procedure:

1. Place three sterile Petri dishes on your desk in an inverted position and mark 1:10, 1:100, and 1:1000.
2. Shake the sample of milk vigorously at least 25 times to obtain a uniform distribution of organisms.
3. Pipette 10 cc. of the milk into a sterile test tube and set aside.
4. Pipette 1 cc. of the milk into a 9-cc. water blank.
5. Mix thoroughly by aspirating and expelling several times with the pipette.
6. With the same pipette, transfer 1 cc. of the dilution to the Petri dish marked 1:10 and 1 cc. to another 9-cc. water blank.
7. Mix in the same manner as given under (5).
8. Transfer 1 cc. of the dilution to the Petri dish marked 1:100 and 1 cc. to the third 9-cc. water blank.
9. Again mix thoroughly.
10. Transfer 1 cc. of the dilution to the Petri dish marked 1:1000.
11. Melt three tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
12. Allow the agar to cool to a temperature of about 45°C.
13. Pour the melted and cooled agar into the Petri dishes.
14. Mix thoroughly by tilting and rotating the dishes.
15. When the agar has solidified, invert the plates and incubate at 37°C. for 48 hr.
16. Only those plates showing from 30 to 300 colonies should be counted. If there are no plates showing colonies within these limits, the plate that comes the nearest to 300 should be counted. Dishes showing less than 20 colonies should not be counted unless it happens that no other plates have been prepared.
17. Heat a water bath to 60°C. and control the flame so that the water remains as close to this temperature as possible.
18. Place the tube containing the 10-cc. sample of milk into the water bath and allow to remain for 30 min.
19. Shake the tube occasionally to prevent the formation of a scum on the surface.
20. At the end of 30 min. remove the tube from the water bath and quickly cool in a pan of cold water. Do not allow the cotton stopper to become wet.
21. Repeat the procedure for the preparation of dilutions and agar plates as given above for the raw milk.
22. Invert all plates and incubate at 37°C. for 48 hr.
23. Prepare Gram stains from the different colonies appearing on the plates of the raw and pasteurized milk.

24. Record your results in the following table:

RAW MILK

	Dilution		
	1:10	1:100	1:1000
Count per cc.			
Gram reaction and morphology			

PASTEURIZED MILK

Count per cc.			
Gram reaction and morphology	-		
Percentage reduction after pasteurization			

Questions:

1. Are all vegetative cells destroyed in the pasteurization process?

2. If a cow had tuberculosis of the lungs, would the organisms appear in the milk?

3. Why does the percentage of reduction in the bacterial count after pasteurization show some variation from one day to another?

4. Why is the holder method generally preferred to the flash method for the pasteurization of milk?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 74

THE BACTERIOLOGY OF BUTTER

Butter prepared from sweet, unpasteurized cream contains the same microflora as the cream from which it was prepared. Butter prepared from cream previously pasteurized at high temperatures, and then inoculated, generally contains only those organisms which were added to promote ripening. Some molds and yeasts may be present that result

from air contamination. Since molds are strongly aerobic, they grow only on the surface of butter.

Many of the organisms responsible for defects in butter are present as a result of contamination after its manufacture. Therefore, the same precautions used in handling milk and cream apply in handling butter. The extent of contamination is roughly an indication of the care exercised in handling the butter.

Required:

    1. Sample of butter.
    2. One 99-cc. water blank.
    3. Three 9-cc. water blanks.
    4. 3 tubes of nutrient agar.
    5. 3 sterile Petri dishes.
    6. 4 sterile 1-cc. pipettes.

Procedure:

    1. Melt the sample of butter in a water bath regulated to a temperature of 37°C.
    2. Warm the water blanks to the same temperature.
    3. Melt three tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.
    4. Allow the agar to cool to a temperature of 50°C.
    5. While the agar is cooling, pipette 1 cc. of the melted butter into the warmed 99-cc. water blank.
    6. Shake thoroughly to obtain an uniform suspension of the organisms.
    7. Remove 1 cc. of the 1:100 dilution and transfer to a warmed 9-cc. water blank.
    8. Mix by rotating the tube between the palms of the hands.
    9. Transfer 1 cc. of the 1:1000 dilution to a Petri dish and 1 cc. to a second 9-cc. water blank.
    10. Mix the contents of the tube as before.
    11. Transfer 1 cc. of the 1:10,000 dilution to a second Petri dish and 1 cc. to a third 9-cc. water blank.
    12. Again mix the contents of the tube.
    13. Transfer 1 cc. of the 1:100,000 dilution to the remaining Petri dish.
    14. Pour the melted agar into the Petri dishes and mix thoroughly by tilting and rotating the plates.
    15. When the agar has solidified, invert the plates and incubate at 37°C. for 48 hr
    16. Count the colonies appearing on the plates. Only those plates showing from 30 to 300 colonies should be counted. If there are no plates showing colonies within these limits, the plate that comes the nearest to 300 should be counted. Dishes showing less than 20 colonies should not be counted unless it happens that no other plates have been prepared.
    17. Calculate the bacterial count per cubic centimeter of butter from the dilution showing the required number of colonies on the plates. If two or more plates show the required number of colonies, the count is determined by taking an average of the results.
    18. Prepare Gram stains from the different colonies appearing on the surface of the agar plates.
    19. Examine under the oil-immersion objective.
    20. Record your results in the following table:

Dilution	Count per gm. butter	Gram stain and morphology
1:1000		
1:10,000		
1:100,000		
Average		

Questions:

1. Why should the butter not be melted at temperatures above 37°C.?
2. Which organism or organisms are commonly employed in the artificial souring of cream?
3. What organism is usually responsible for the natural souring of cream?
4. What is the difference between a starter and a culture?
5. Under what conditions are pathogenic organisms likely to be present in butter?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 75

THE BACTERIOLOGY OF ICE CREAM

The bacterial content of ice cream depends largely upon the number present in the cream at the time of preparation. Sweet pasteurized cream handled in a hygienic manner contains relatively few organisms. On the other hand, old cream or cream not stored at low temperatures may show very high bacterial counts. The presence of psychrophilic organisms in ice cream may result in an increase in numbers. The lactic acid organisms fail to multiply at low temperatures. The presence of pathogenic organisms in ice cream is usually the result of using contaminated cream in its manufacture.

Required:

1. Sample of ice cream.
2. One 99-cc. water blank.
3. Three 9-cc. water blanks.
4. 4 tubes of nutrient agar.
5. 2 sterile 1-cc. pipettes.
6. 4 sterile Petri dishes.
7. Slides.

Procedure:

    1. Allow the sample of ice cream to melt at room temperature.

    2. Transfer 1 cc. of the melted sample to a 99-cc. water blank.

    3. Mix thoroughly.

    4. With a fresh pipette, transfer 1 cc. of the 1:100 dilution to a sterile Petri dish and 1 cc. to a 9-cc. water blank.

    5. Mix the contents of the tube by aspirating and expelling several times with the pipette.

    6. Transfer 1 cc. of the 1:1000 dilution to a second Petri dish and 1 cc. to a second 9-cc. water blank.

    7. Mix thoroughly as before.

    8. Transfer 1 cc. of the 1:10,000 dilution to a third Petri dish and 1 cc. to the remaining 9-cc. water blank.

    9. Mix thoroughly.

    10. Transfer 1 cc. of the 1:100,000 dilution to the fourth Petri dish.

    11. Melt four tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

    12. Allow the agar to cool to a temperature of 45°C.

    13. Pour the melted and cooled agar into the Petri dishes.

    14. Mix thoroughly by tilting and rotating the dishes.

    15. When the agar has solidified, invert the plates and incubate at 37°C. for 48 hr.

    16. Only those plates showing from 30 to 300 colonies should be counted. If there are no plates showing colonies within these limits, the plate that comes the nearest to 300 should be counted. Plates showing less than 20 colonies should not be counted unless it happens that no other plates have been prepared.

    17. Prepare Gram stains from the different colonies and examine under the oil-immersion objective.

    18. Record your results in the following table:

Dilution	Count	Gram stain and morphology
1:100		
1:1000		
1:10,000		
1:100,000		
Average		

Questions:

1. What are the causes of high bacterial counts in ice cream?
2. What organism would you expect to predominate in ice cream prepared from fresh, clean cream?
3. Is ice cream a good culture medium for the growth of bacteria?

Reference

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 76

THE MICROBIOLOGY OF CHEESE

Many organisms are responsible for the aromas, flavors, and characteristics of the various kinds of cheeses. Each type has its own characteristic flora. Some of the bacterial organisms that have been isolated include *Streptococcus lactis, S. cremoris, Leuconostoc citrovorum, L. dextranicum, S. thermophilus, Lactobacillus casei, L. lactis, L. bulgaricus, L. helveticus, L. plantarum,* and *Propionibacterium shermanii.*

In addition several species of molds, especially those of the genus *Penicillium,* have been isolated from certain types of cheeses. Since these organisms are strongly aerobic, they occur either on the surface or along cracks in the cheese.

Required:

1. Samples of various types of cheeses.
2. Sterile mortar and pestle.
3. One 99-cc. water blank.
4. Two 9-cc. water blanks.
5. 3 tubes of nutrient agar.
6. 3 sterile Petri dishes.
7. 3 sterile 1-cc. pipettes.
8. Slides.

Procedure:

1. Weigh out about 1 gm. of cheese on a piece of sterile paper. Use sterile instruments for cutting and handling the cheese.
2. Record the weight in your notebook.
3. Place the cheese in a sterile mortar and add aseptically a few cubic centimeters of water from a 99-cc. water blank.
4. Rub the cheese with a sterile pestle until a uniform suspension is obtained.
5. Observe aseptic precautions throughout.
6. Pour the contents of the mortar into the same 99-cc. water blank and rinse out the mortar with a few cubic centimeters of the suspension.
7. Mix thoroughly.
8. Transfer 1 cc. of the 1:100 suspension to a sterile Petri dish and 1 cc. to a 9-cc. water blank.
9. Mix well by rotating the tube between the palms of the hands.

10. Transfer 1 cc. of the 1:1,000 suspension to a second Petri dish and 1 cc. to another 9-cc. water blank.

11. Mix thoroughly.

12. Transfer 1 cc. of the 1:10,000 suspension to a third Petri dish.

13. Melt three tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

14. Allow the agar to cool to a temperature of 45°C.

15. Pour the melted and cooled agar into the Petri dishes.

16. Mix thoroughly by tilting and rotating the plates.

17. When the agar has solidified, invert the plates and incubate at 37°C. for 48 hr.

18. Count the colonies appearing on the plates and calculate the number of organisms per gram of cheese.

19. Store the plates in your locker for 1 week.

20. Prepare Gram stains from the different colonies appearing on the plates, including molds and yeasts.

21. Record your results in the following table:

Dilution	Count	Gram stain and morphology
1:100		
1:1000		
1:10,000		
Average		

Questions:

1. Are cheese counts as high as those of milk and butter?   Why?
2. What is the morphology of the predominating organism?
3. Why are the plates set aside for 1 week before preparing Gram stains?
4. Name at least two molds that are important in the manufacture of cheese.

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIOLOGY OF FOOD

EXERCISE 77

THE MICROBIOLOGY OF SWEETENED CONDENSED MILK

Sweetened condensed milk is not sterilized after being placed in cans. The high osmotic pressure of the preparation, due to the added sugar, is sufficient to prevent multiplication of most organisms. However, only a small percentage of cans are ever sterile. The most important organisms that have been isolated include staphylococci, streptococci, anaerobes, gas-producing rods, aerobic spore-forming bacilli; and thermophilic forms. In addition to these, molds and yeasts are commonly encountered.

Yeasts are probably the most common organisms found in sweetened condensed milk. They are active fermenters, attacking the lactose of the milk or the added sucrose or both with the liberation of gas. This results in the cans having a blown appearance.

Required:

1. Can of blown sweetened condensed milk.
2. 2 tubes of glucose agar.
3. 1 tube of glucose brain medium.
4. 2 sterile Petri dishes.
5. 1 sterile test tube.
6. Sterile 1-cc. pipettes.
7. 95 per cent alcohol.
8. Sterile can opener.
9. Slides.

Procedure:

1. Pour several drops of alcohol over the top of the can of milk.
2. Burn off the alcohol to sterilize the top.
3. Punch a hole in the sterilized top with a sterile can opener. Make sure the hole is large enough to insert a 1-cc. pipette.
4. Cover the top of the can with the lid of a sterile Petri dish.
5. Remove some of the milk with a sterile 1-cc. pipette and place 1 drop on each of two glucose agar plates.
6. With the wire loop, streak the milk over the surface of the plates.
7. Incubate one plate at 37°C. and the other at 55°C. for 48 hr.
8. Pipette a few cubic centimeters of the milk into a sterile test tube.
9. Place the tube in a pan of water previously heated to 80°C. and hold at this temperature for 10 min.
10. Pipette 0.5 cc. of the heated milk into a tube of brain medium and mix thoroughly.

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11. Incubate the tube at 37°C. for 48 hr.

12. Prepare Gram stains from the different types of colonies appearing on the plates.

13. Prepare a Gram stain from the tube of brain medium.

14. Record your results in the following table:

| Colony | Temperature 37°C. | | |
	Colonial characteristics	Pigment	Gram reaction and morphology
1			
2			
3			
4			
Brain medium			
Temperature 55°C.			
Colony 1			
2			

Questions:

1. Why is the milk heated to 80°C. for 10 min. previous to inoculation into brain medium?

2. Does the presence of growth in the brain medium indicate anaerobic multiplication only?

3. Are yeasts aerobic or anaerobic?

4. Why are cans of sweetened condensed milk not sterilized?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 78

THE BACTERIOLOGY OF MEAT

Many species of aerobic and anaerobic organisms have been isolated from meat. These include Gram-positive, aerobic, spore-bearing rods of the *Bacillus subtilis* type; Gram-negative, aerobic nonspore-forming rods of the *Escherichia*, *Aerobacter*, and *Proteus* genera; staphylococci, micrococci, and sarcinae; anaerobes of the genus *Clostridium;* and yeasts and molds. The most pronounced changes in meats are produced by the anaerobic, spore-bearing organisms. These organisms are responsible for putrefactive changes on proteins resulting in the liberation of foul-smelling compounds.

There appears to be no correlation between the bacterial population of meat and its sanitary quality. It is not so much the numbers as it is the kinds of organisms that determine the sanitary quality of meat.

Required:

1. Sample of ground meat.
2. 6 tubes of nutrient agar.
3. One 99-cc. water blank.
4. Three 9-cc. water blanks.
5. Sterile sand.
6. Sterile papers for weighing.
7. Sterile spatula.
8. Sterile mortar and pestle.
9. 6 sterile Petri dishes.
10. 2 sterile 10-cc. pipettes.
11. 4 sterile 1-cc. pipettes.
12. Slides.

Procedure:

1. Weigh out 1 gm. of ground meat on a square of sterile paper. Use a sterile spatula for handling the meat.

2. Transfer the weighed sample to a sterile mortar containing about 5 gm. of sterile sand.

3. Remove about 5 cc. of water from a 99-cc. water blank and transfer to the mortar.

4. Triturate the meat thoroughly with a pestle until it is reduced to very small particles.

5. Transfer 10 cc. more of water from the 99-cc. water blank to the mortar and mix thoroughly.

6. Pour the contents of the mortar into the 99-cc. water blank.

7. Repeat this procedure of pouring from flask to mortar and back again, two more times. Observe aseptic precautions.

8. This gives a 1:100 dilution of the original sample of meat.

9. Mix thoroughly to obtain a uniform suspension of the organisms.

10. Transfer immediately 1 cc. of the 1:100 dilution to a 9-cc. water blank.

11. Mix thoroughly by rotating the tube between the palms of the hands.

12. With a fresh pipette, transfer 1 cc. of the 1:1000 dilution to each of two sterile Petri dishes and 1 cc. to a second 9-cc. water blank. Mix thoroughly.

13. Transfer 1 cc. of the 1:10,000 dilution to each of two sterile Petri and 1 cc. to a third 9-cc. water blank. Mix thoroughly.

14. Transfer 1 cc. of the 1:100,000 dilution to each of two sterile Petri dishes.

15. Melt six tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

16. Allow the agar to cool to a temperature of 45°C.

17. Pour the melted and cooled agar into the Petri dishes.

18. Mix by tilting and rotating the dishes.

19. When the agar has hardened, invert the plates and incubate at 37°C. for 48 hr.

20. Discard the plates showing more than 300 colonies.

21. Count the remaining plates and calculate the number of organisms per gram of meat.

22. Prepare Gram stains from the different kinds of colonies appearing on the plates.

23. Record your results in the following table:

Dilution	Count per plate	Count per gram of meat	Gram stain and morphology
1:1000	1.		
	2.		
1:10,000	1.		
	2.		
1:100,000	1.		
	2.		

Average count per gram of meat

Questions:

1. Is it easy to obtain representative samples of ground meats for analyses?
2. Do different kinds of meats have their own characteristic floras?
3. Why is an incubation temperature of 28°C. preferable to 37°C. for the examination of meats?
4. Why do bacterial counts fail to correlate with the sanitary quality of meats?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 79

THE BACTERIOLOGY OF DRIED BEEF

Dried beef is prepared by pickling meat in brine, soaking in water to remove excess salt, then smoking and drying to remove most of the moisture. In this condition dried beef may be preserved for long periods of time.

Since dried beef is impregnated with salt and the outside treated with creosote and other compounds present in wood smoke, it may be preserved almost indefinitely without any decomposition. The high osmotic pressure and low moisture content is unfavorable to bacterial growth. Therefore, the bacterial content of dried beef is considerably less than that of fresh meat.

Required:

1. Sample of dried beef.
2. 4 tubes of nutrient agar.
3. Sterile papers for weighing.
4. Sterile spatula.
5. Sterile meat grinder.
6. Sterile sand.
7. Sterile mortar and pestle.
8. One 99-cc. water blank.
9. Two 9-cc. water blanks.
10. 5 sterile Petri dishes.
11. 2 sterile 1-cc. pipettes.
12. 2 sterile 10-cc. pipettes.
13. Slides.

Procedure:

1. Pass the dried beef through a sterile meat grinder and collect the meat in a sterile Petri dish.
2. With a sterile spatula, weigh out 1 gm. of the ground beef on a square of sterile paper.
3. Transfer the weighed sample to a sterile mortar containing about 5 gm. of sterile sand.
4. Remove about 5 cc. of water from a 99-cc. water blank and transfer to the mortar.

5. Triturate the meat thoroughly with a pestle until it is reduced to very small particles.

6. Transfer 10 cc. more of water from the 99-cc. water blank to the mortar and mix thoroughly.

7. Pour the contents of the mortar into the 99-cc. water blank.

8. Repeat this procedure of pouring from flask to mortar and back again, two more times. Observe aseptic precautions.

9. This gives a 1:100 dilution of the original sample of meat.

10. Mix thoroughly to obtain a uniform suspension of the organisms.

11. Transfer immediately 1 cc. of the 1:100 dilution to a 9-cc. water blank.

12. Mix thoroughly by rotating the tube between the palms of the hands.

13. With a fresh pipette, transfer 1 cc. of the 1:1000 dilution to each of two sterile Petri dishes and 1 cc. to a second 9-cc. water blank. Mix thoroughly.

14. Transfer 1 cc. of the 1:10,000 dilution to each of two sterile Petri dishes.

15. Melt four tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

16. Allow the agar to cool to a temperature of 45°C.

17. Pour the melted and cooled agar into the Petri dishes.

18. Mix by tilting and rotating the dishes.

19. When the agar has hardened, invert the plates and incubate at 37°C. for 48 hr.

20. Discard the plates showing more than 300 colonies.

21. Count the remaining plates and calculate the number of organisms per gram of meat.

22. Prepare Gram stains from the different kinds of colonies appearing on the plates.

23. Record your results in the following table:

Dilution	Count per plate	Count per gram meat	Gram stain and morphology
1:1000	1.		
	2.		
1:10,000	1.		
	2.		

Average count per gram of meat

Questions:

1. Does dried beef show a higher or a lower count than fresh beef?
2. Do bacteria multiply on dried beef?
3. Do the antiseptic compounds present in wood smoke penetrate meat to any considerable distance?
4. Why is the meat pickled before being dried?
5. Why is saltpeter added to the pickling solution?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 80

THE BACTERIOLOGY OF EGGS

Fresh eggs are not always free from microorganisms. The organisms that have been isolated include various species of cocci, bacilli, and molds. The bacteriology and mycology of eggs have not been studied sufficiently to give an accurate picture of the flora of contaminated eggs.

An eggshell is porous in structure. The pores are sufficiently large to permit the passage of gases and microscopic particles. Organisms are always present on the shells of eggs. *Escherichia coli* is present on practically every shell. Damp and soiled eggs soon become contaminated owing to the fact that the moisture permits the passage of organisms through the pores. Therefore, eggs that are to be kept for some time should never be washed in water.

Required:

1. 24-hr. nutrient broth culture of *Escherichia coli*.
2. 24-hr. nutrient broth culture of *Proteus vulgaris*.
3. 2 fresh sound eggs.
4. 1 tube of lactose fermentation broth with bromothymol blue indicator.
5. 1 tube of sucrose fermentation broth with bromothymol blue indicator.
6. 500 cc. of sterile nutrient broth.
7. 1 eosin methylene blue (E.M.B.) agar plate.
8. 1 tube of nutrient gelatin.
9. 0.1 per cent solution of mercuric chloride.
10. 95 per cent alcohol.
11. 200 cc. sterile water.
12. 4 sterile pint-size canning jars with caps.
13. 2 sterile Petri dishes.
14. 2 sterile 600-cc. beakers.
15. Two 600-cc. beakers.
16. Spatula.
17. Stiff hand brush.
18. Granite spoon.
19. Slides.

Procedure :

1. Contaminated eggs may be prepared in the following manner: Wash two sound eggs thoroughly in water by scrubbing them with a stiff brush. Immerse the eggs for 1 hr. in a 0.1 per cent solution of mercuric chloride contained in a beaker. Remove from the mercuric chloride solution and transfer to a beaker of sterile water. A large granite spoon may be used for handling the eggs. Immerse the eggs for 10 min. in 95 per cent alcohol contained in a beaker.

2. In the meantime pour 250 cc. of nutrient broth into each of two sterile pint-size canning jars and quickly replace the caps.

3. Inoculate one jar with a loopful of a 24-hr. nutrient broth culture of *E. coli.*

4. Inoculate the other jar with a loopful of a 24-hr. nutrient broth culture of *P. vulgaris.*

5. Remove the eggs from the alcohol and place in a sterile Petri dish cover.

6. Burn off the alcohol remaining on the shells.

7. Place one egg in the broth inoculated with *E. coli* and the other in the broth inoculated with *P. vulgaris.*

8. Replace the caps and incubate the jars at 37°C. for 4 days.

9. Remove the eggs from the jars and immerse in a 0.1 per cent solution of mercuric chloride for 30 min.

10. Remove from the mercuric chloride solution and transfer to a beaker of sterile water.

11. Immerse the eggs in 95 per cent alcohol for 10 min.

12. Burn off the alcohol remaining on the shells.

13. Sterilize a spatula by passing it several times through the flame of a Bunsen burner.

14. Grasp the large end of the egg with the left hand and crack the shell through the center with the spatula.

15. Carefully pull apart both halves of the shell and allow the egg to fall into a sterile canning jar. Do not let your fingers touch the egg to avoid external contamination.

16. Mix the yolk and white thoroughly.

17. Do likewise with the other egg.

18. Inoculate a tube of lactose fermentation broth with about 0.5 cc. of the mixed egg previously immersed in a culture of *E. coli.*

19. Inoculate a tube of sucrose fermentation broth with about 0.5 cc. of the mixed egg previously immersed in a culture of *P. vulgaris.*

20. Incubate both tubes at 37°C. for 48 hr.

21. If the tubes show acid and gas, prepare Gram stains.

22. The Gram stains should show pure cultures of Gram-negative, nonsporulating rods.

23. Streak an E.M.B. agar plate with one loopful of the lactose broth culture of *E. coli.*

24. Stab a tube of nutrient gelatin with one loopful of the sucrose broth culture of *P. vulgaris.*

25. Incubate the plate and tube of gelatin at 37°C. for 48 hr.

26. Record your results in the following table:

Character	Culture	
	E. coli	*P. vulgaris*
Gram reaction and morphology		
Lactose broth		
E.M.B. agar		
Sucrose broth		
Nutrient gelatin		

Questions:

1. What do you conclude from this experiment?

2. Why is it necessary to immerse eggs in 0.1 per cent mercuric chloride for one hour to éffect sterilization?

3. Do eggs furnish a good culture medium for the growth of organisms?

4. Which show a higher percentage of contaminations, fertile or nonfertile eggs?

5. Why do the yolks of boiled eggs sometimes appear black?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIOLOGY OF SOIL

EXERCISE 81

THE QUANTITATIVE EXAMINATION OF SOIL BY THE AGAR PLATE METHOD

The enumeration of the number of organisms in soil by this procedure presents several serious objections. Some of these objections are (1) Anaerobes fail to grow on the surface of agar exposed to air, (2) autotrophic bacteria do not multiply on an organic medium, (3) nonsymbiotic nitrogen-fixing cells grow only to a limited extent, (4) many of the cellulose-digesting forms fail to grow on nutrient agar, (5) sulfate reducers require the presence of sulfates and a satisfactory oxidizable organic compound. The counts represent only a fraction of the total bacterial population of the soil. However, the procedure does give an accurate index of the number of organisms present in the soil which are able to grow on nutrient agar.

Required:

1. Samples of soils collected from different localities and at varying depths.
2. 5 tubes of nutrient agar.
3. One 99-cc. water blank.
4. Four 9-cc. water blanks.
5. 5 sterile Petri dishes.
6. Sterile mortar and pestle.
7. Sterile squares of paper.
8. Sterile spatula.
9. 5 sterile 1-cc. pipettes.
10. Slides.

Procedure:

1. Place some of the soil in a sterile mortar and break up the lumps with a pestle.
2. Weigh out 1 gm. of the soil on a square of sterile paper. Handle the soil with a sterile spatula.
3. Transfer the weighed sample of soil to a sterile 99-cc. water blank.
4. Shake thoroughly to obtain a uniform suspension of organisms.
5. Transfer 1 cc. of the 1:100 dilution to a sterile Petri dish and 1 cc. to a 9-cc. water blank.
6. Mix thoroughly by rotating the tube between the palms of the hands.
7. Transfer 1 cc. of the 1:1000 dilution to another Petri dish and 1 cc. to a second 9-cc. water blank.

8. Mix the contents of tl   water blank thoroughly as before.

9. In a similar manner prepare dilutions of 1:100,000 and 1:1,000,000 and transfer 1-cc. amounts to sterile Petri dishes.

10. Melt five tubes of nutrient agar in an Arnold sterilizer or in a pan of boiling water.

11. Cool the agar to a temperature of 50°C.

12. Pour the melted and cooled agar into the Petri dishes and mix thoroughly by tilting the plates from side to side.

13. When the agar has hardened, invert the plates and incubate at 20°C. for 1 week.

14. Count only those plates showing from 30 to 300 colonies.

15. Calculate the count per gram of soil.

16. Record your results in the following table:

Soil sample	Cultivated, uncultivated	Depth, ft.	Dilution	Count per plate
			1:100	
			1:1000	
			1:10,000	
			1:100,000	
			1:1,000,000	

Average count per gram of soil =

Questions:

1. Do soil counts increase or decrease with depth?

2. Why is a temperature of 20°C. rather than 37°C. used?

3. Why is agar used in preference to gelatin for soil counts?

4. Does soil furnish a good culture medium for the growth of organisms?

5. Why is the agar plate method unsatisfactory for the growth of all species of organisms found in soil?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 82

THE QUANTITATIVE EXAMINATION OF SOIL BY THE DIRECT MICROSCOPIC METHOD

The direct microscopic method is considered more accurate than the agar plate method for determining the total number of organisms in soil. It is not necessary to cultivate the organisms first as is necessary in the agar plate method, thereby eliminating errors due to failure of certain species to grow in nutrient agar.

On the other hand, the agar plate method possesses certain advantages over the direct procedure. Dead organisms are not enumerated whereas the direct method fails to distinguish dead organisms from living. Other sources of error in the direct method include failure to prepare uniform smears, and difficulty in distinguishing bacteria from soil particles.

It may be concluded that neither method is satisfactory for enumerating the total viable population of the soil.

Required:

1. Samples of the same soils used in Exercise 81.
2. One 99-cc. dilution blank consisting of a 0.015 per cent solution of agar in water.
3. 1 per cent solution of rose bengal.
4. Sterile mortar and pestle.
5. Sterile squares of paper.
6. Sterile spatula.
7. Piece of cardboard having a ruled area of 4 sq. cm. (1 by 4 cm.).
8. Sterile 0.1-cc. pipette.
9. Slides.

Procedure:

1. Place some of the soil in a sterile mortar and break up the lumps with a pestle.
2. Weigh out 10 gm. of the soil on a square of sterile paper. Handle the soil with a sterile spatula.[1]
3. Transfer the weighed sample of soil to a sterile 99-cc. dilution blank consisting of an 0.015 per cent solution of agar in water.
4. Shake thoroughly to obtain a uniform suspension of organisms.
5. Pass a clean slide through the flame of a Bunsen burner to remove any grease present.
6. Place the slide over the piece of cardboard and follow the ruled area with a china marking pencil. An area of approximately 4 sq. cm. is now ruled off on the slide. Do not allow your fingers to touch the ruled area!

[1] This should make sufficient soil suspension for the needs of the entire class.

7. Shake the soil suspension and immediately transfer 0.1 cc. to the center of the ruled slide.

8. By means of your wire loop, spread the suspension uniformly over the ruled area.

9. If the liquid does not spread uniformly, it indicates a greasy slide. As it is useless to continue, start with another slide.

10. Allow the slide to dry on a flat surface over a boiling water bath.

11. When dry and while still on the water bath, cover the film with a few drops of rose bengal solution.

12. Allow the film to stain for 1 min. Sufficient solution should be added to prevent drying during the staining period.

13. Wash the slide very briefly by immersion in water.

14. Drain the slide, blot, and air-dry.

15. Examine the slide under the oil-immersion objective. Count 25 fields from different areas of the slide and compute the average number of organisms per field.

16. Having determined the area of the field of the oil-immersion objective (Exercise 4), calculate the number of organisms present on the entire slide.

17. This gives the number of organisms present in the 0.1 cc. of suspension. From this it is a simple matter to calculate the count per gram of soil.

18. Record your results in the following table:

Soil sample	Average count per field	Area of each field	Count on entire slide	Count per gram soil

Compare the counts with those obtained from the same soil by the method given in the preceding exercise.

Questions:

1. Why are counts by the direct method higher than by the agar plate method?

2. Why is agar used in the dilution blank?

3. Could gelatin be substituted for the agar?

4. Discuss some precautions to be observed in preparing satisfactory stained preparations.

5. Does rose bengal act better in an acid or an alkaline solution? Why?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 83

AMMONIFICATION BY BACTERIA

Ammonification may be defined as the release of ammonia from various nonprotein compounds as distinguished from deaminization which means the release of ammonia following the hydrolysis of proteins or protein split products, such as proteoses, peptones, peptids, and amino acids.

Stable and barnyard manures are often used as fertilizers. They help to replenish the nitrogen supply of the soil. A high content of urine is often present in such waste material. The most important nitrogen compound present in urine is urea. Many soil organisms have the power of decomposing urea with the liberation of ammonia.

The production of ammonia is the first stage in the formation of nitrates in the soil.

Required:

    1. 48-hr. urea broth culture of *Micrococcus ureae,* incubated at 25°C.

    2. 48-hr. urea broth culture of *Bacillus freudenreichii,* incubated at 25°C.

    3. 48-hr. urea broth culture of *Pseudomonas ureae,* incubated at 25°C.

    4. 48-hr. urea broth culture of *Sarcina ureae,* incubated at 25°C.

    5. 5 tubes of Kappen's urea broth.

    6. 0.02 per cent solution of phenol red indicator.

    7. Phenol red standards, covering the range from pH7.0 to 8.6 at intervals of 0.2pH.

    8. Comparator block.

    9. Test tubes for the comparator block.

    10. Five 10-cc. pipettes.

    11. 1-cc. pipette.

    12. Slides.

Procedure:

    1. Inoculate a tube of Kappen's urea broth with one loopful of a 48-hr. broth culture of *M. ureae.*

    2. Inoculate a second tube with one loopful of a 48-hr. broth culture of *B. freudenreichii.*

    3. Inoculate a third tube with one loopful of a 48-hr. broth culture of *S. ureae.*

    4. Inoculate a fourth tube with one loopful of a 48-hr. broth culture of *P. ureae.*

    5. The last tube serves as a control.

    6. Incubate the tubes at 25°C. for 4 days. If a 25°C. incubator is not available, store the tubes in your laboratory locker.

    7. At the end of the incubation period determine the pH of the cultures and control tube in the following manner:

    8. Pipette 3 cc. of one of the cultures into each of two test tubes.

    9. Add 0.3 cc. phenol red indicator to only one of the tubes. Mix thoroughly.

    10. If the culture is acid or neutral, the color will be yellow or orange; if alkaline it will be red.

    11. Set up a comparator block as shown in Exercise 17.

    12. Try different pH color standards until one is found that matches the color of the tube containing culture with indicator.

    13. The pH reading on the color standard gives the approximate hydrogen-ion concentration of the urea broth culture under examination.

    14. Examine the remaining cultures and control tube in the same manner.

    15. Prepare Gram stains of the various cultures and examine under the oil-immersion objective.

    16. Record your results in the following table:

Culture	pH	Spores	Gram reaction and morphology
M. ureae			
B. freudenreichii			
S. ureae			
P. ureae			
Control			

Questions:

1. How would you proceed to isolate urea-decomposing organisms from manure?
2. Why do the organisms attack urea?
3. What might happen if you added a small amount of an easily available carbohydrate to the medium?
4. What importance is attached to the decomposition of urea in the soil?
5. Are bacteria capable of producing urea?

Reference

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 84

NITROSIFICATION BY BACTERIA

Certain autotrophic species obtain their energy from the oxidation of ammonia to nitrite. The process is commonly known as nitrosification. The organisms involved are members of the genera *Nitrosomonas* and *Nitrosococcus*.

The organisms grow poorly on the usual organic media but may be easily cultivated on an inorganic medium containing an ammonium salt. Since the organisms are strongly aerobic, they grow best in culture media exposed to air in shallow layers.

Required:

1. Sample of fertile soil having a neutral or alkaline reaction.

2. 500-cc. flask containing 100 cc. of Winogradsky's ammonium sulfate solution without magnesium carbonate.

3. Sterile 10 per cent aqueous suspension of magnesium carbonate.

4. Sulfanilic acid test solution.

5. α-Naphthylamine test solution.

6. Paper for weighing.

7. Spatula.

8. Test tubes.

9. Slides.

Procedure:

1. Shake the sterile 10 per cent suspension of magnesium carbonate and transfer 10 cc. to a flask containing 100 cc. of Winogradsky's medium.

2. Weigh out 1 gm. of the soil on a square of paper.

3. Transfer the soil to the flask of medium and mix well.

4. Incubate the flask at a temperature of 25°C. If a 25°C. incubator is not available, place the flask in your laboratory locker.

5. At the end of 4 days and every 2 days thereafter carefully remove 1 cc. of the culture and transfer to a test tube.

6. Add 2 drops of sulfanilic acid test solution and 2 drops of α-naphthylamine test solution to the culture in the tube.

7. The presence of nitrite is indicated by the appearance of a pink or red color.

8. An incubation period of 2 or 3 weeks may be required for a strong nitrite test.

9. When the test is strongly positive, prepare a Gram stain and examine the slide under the oil-immersion objective.

10. Record your results in the following table:

Soil sample	Positive nitrite test, days	Gram reaction and morphology

Questions:

1. Why should the sample of soil have a neutral or alkaline reaction?

2. Why should an excess of magnesium carbonate be present in the medium?

3. Why is the magnesium carbonate sterilized separately?

4. Write an equation for the reaction.

5. What is an elective medium?

6. Is Winogradsky's medium considered elective?

7. Do the ammonia-oxidizing organisms produce a pellicle?

Reference

Salle, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

Exercise 85

NITRIFICATION BY BACTERIA

Several autotrophic species obtain their energy from the oxidation of nitrites to nitrates. The process is commonly referred to as nitri-

fication. The organisms concerned are members of the genus *Nitrobacter*.

The medium employed for the cultivation of the organisms is similar to that used in the preceding exercise except that sodium nitrite is substituted for the ammonium sulfate. Since the organisms are obligately aerobic, they grow best in culture media exposed to air in shallow layers. Pure colonies may be obtained by streaking a loopful of the liquid culture over the surface of the same medium solidified with agar.

Required:

1. Sample of fertile soil having a neutral or alkaline reaction.
2. 500-cc. flask containing 100 cc. of sodium nitrite medium.
3. Sulfanilic acid test solution.
4. α-Naphthylamine test solution.
5. Diphenylamine test solution.
6. Concentrated sulfuric acid.
7. Paper for weighing.
8. Spatula.
9. Test tubes.
10. Slides.

Procedure:

1. Weigh out 1 gm. of the soil on a square of paper.
2. Transfer the soil to the flask of medium and mix well.
3. Incubate the flask at a temperature of 25°C. If a 25°C. incubator is not available place the flask in your laboratory locker.
4. At the end of 4 days and every 2 days thereafter carefully remove 1 cc. of the culture and transfer to a test tube.
5. Add 2 drops of sulfanilic acid test solution and 2 drops of α-naphthylamine test solution to the culture in the tube.
6. The presence of nitrite is indicated by the appearance of a pink or red color.
7. Continue the tests for nitrite at intervals of 2 days until the reaction is negative, *i.e.*, no color is obtained.
8. This indicates that the nitrite has been completely oxidized to nitrate.
9. When the nitrite test is negative, remove 1 cc. of the culture and transfer to a test tube.
10. Add 2 drops of diphenylamine test solution and 2 drops of concentrated sulfuric acid. A blue color indicates the presence of nitrate.
11. The test is positive for both nitrite and nitrate but since the nitrite test was negative above, the blue color must indicate nitrate only.
12. When the test is strongly positive for nitrate, prepare a Gram stain and examine the slide under the oil-immersion objective.
13. Record your results in the following table:

Soil sample	Negative nitrite test, days	Gram reaction and morphology

Questions:

1. Why should the sample of soil have a neutral or alkaline reaction?
2. Why is the sodium carbonate added to the medium?
3. Write an equation for the reaction.
4. Do the organisms produce a pellicle on the medium?
5. Is sodium nitrite medium considered elective?
6. Are the various species Gram-positive or Gram-negative?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 86

THE OXIDATION OF THIOSULFATES BY BACTERIA

The genus *Thiobacillus* includes small rod-shaped, autotrophic organisms capable of oxidizing thiosulfates, sulfides and free sulfur to sulfur, persulfates, and sulfates under acid or alkaline conditions. They obtain their carbon from carbon dioxide or from bicarbonates and carbonates in solution.

In an inorganic medium containing thiosulfate, *Thiobacillus thioparus* is capable of oxidizing the compound to sulfate and free sulfur. A pellicle, consisting of a mixture of bacteria and sulfur granules, forms on the surface. The production of sulfate and free sulfur from thiosulfate is an exothermic reaction resulting in the release of considerable energy.

Required:

1. Sample of fertile soil.
2. Sample of fresh manure.
3. Two 250-cc. Erlenmeyer flasks, each containing 100 cc. of Beijerinck's thiosulfate solution.
4. 2 tubes of Beijerinck's thiosulfate agar.
5. 2 sterile Petri dishes.
6. 2 test tubes.
7. Paper for weighing.
8. Spatula.
9. Sterile 1-cc. pipettes.
10. Slides.

Procedure:

1. Inoculate a flask of Beijerinck's thiosulfate solution with 1 gm. of fertile soil.
2. Inoculate the other flask of the same medium with 1 gm. of fresh manure.
3. Mix the contents of both flasks thoroughly.
4. Incubate the flasks at a temperature of 25 to 30°C. If such an incubator is not available, store the flasks in your laboratory locker.
5. At the end of 4 days remove 1 cc. of medium from each flask and transfer to clean test tubes. Handle the flasks as carefully as possible to avoid destroying the pellicles.

6. Add Gram's iodine solution, drop by drop, to the 1-cc. portions of medium until the color remains permanent. Record the number of drops required.

7. Repeat the above procedure every 2 days until a permanent color is obtained after the addition of only 1 drop of iodine solution.

8. This indicates that all of the thiosulfate has been oxidized to sulfate and free sulfur.

9. Melt two tubes of Beijerinck's thiosulfate agar in an Arnold sterilizer or in a pan of boiling water.

10. Allow the agar to cool to a temperature of about 50°C.

11. Pour the melted and cooled agar into the two sterile Petri dishes.

12. Set the plates aside until the agar has hardened.

13. Remove one loopful of the pellicle from the fertile-soil culture and streak over the surface of one of the agar plates.

14. Remove one loopful of the pellicle from the manure culture and streak over the surface of the other agar plate.

15. Invert the plates and incubate at 25 to 30°C. until sulfur-yellow colonies appear.

16. Prepare Gram stains from typical colonies and examine under the oil-immersion objective.

17. Record your results in the following table:

Culture	Complete oxidation of thiosulfate, days	Gram reaction and morphology
Fertile soil		
Manure		

Questions:

1. Write the equation for the oxidation of thiosulfate to sulfate and free sulfur.
2. Is the reaction aerobic or anaerobic?
3. What compounds are produced when iodine is added to sodium thiosulfate?
4. What use is made of the above reaction?
5. Does this organism accumulate sulfur granules within the cell?
6. Would you expect sewage to be rich in sulfur-oxidizing organisms?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 87

THE DIGESTION OF CELLULOSE

Some organisms obtain their energy from the digestion of cellulose as the only source of carbon. In the absence of the compound the organisms grow very poorly or not at all.

Cellulose is attacked by both aerobic and anaerobic organisms. The majority of the species fall into six recognized genera. In addition, a number of molds are also capable of attacking cellulose as a source of carbon. These latter organisms are commonly observed growing on old paper, especially if kept in a moist atmosphere.

Cellulose-digesting organisms are of tremendous importance in the dissolution of insoluble cellulose in the soil. They are responsible for the removal of the remains of the preceding plant crop, making it available as a food.

The organisms may be easily cultivated in an inorganic medium containing a strip of filter paper as the only source of energy.

Required:

    1. Sample of horse or cow manure.
    2. 24-hr. nutrient broth culture of *Escherichia coli.*
    3. Two tubes of McBeth's cellulose ammonium sulfate solution.
    4. Sterile vaspar.
    5. Slides.

Procedure:

    1. Place a tube of McBeth's cellulose ammonium sulfate solution in an Arnold sterilizer and steam for about 5 min.
    2. Remove from the sterilizer and allow to cool to about 50°C. Do not shake the tube!
    3. Inoculate the tube with about 1 gm. of horse or cow manure.
    4. Inoculate the unheated tube with one loopful of a 24-hr. nutrient broth culture of *E. coli* as a control.
    5. Melt a tube or flask of vaspar in the Arnold sterilizer.
    6. Pour a ½-in. layer of the melted vaspar over the surface of both tubes.
    7. Incubate the manure culture at 55°C.; the *E. coli* culture at 37°C.
    8. Examine the tubes daily. Record when disintegration of the filter paper begins and when dissolution is complete.
    9. Note the following: discoloration of filter paper and evolution of gas.
    10. When dissolution is complete, remove the tube from the incubator.
    11. Gently heat the vaspar seal in the flame of a Bunsen burner to loosen it from the sides of the tube.
    12. Submerge the seal with the inoculating wire so that a loop of the medium can be removed.
    13. Prepare a Gram stain and examine it under the oil-immersion objective.
    14. Record your results in the following table.

Disintegration of paper		Discol- oration	Gas	Gram reaction and morphology
Begin	Complete			

Questions:

1. Why is the tube steamed before being inoculated with the manure?
2. Why is vaspar added?
3. Why are cellulose-digesting organisms present in manure?
4. Why is the tube incubated at 55°C. instead of at 37°C.?
5. What is a cytase?
6. Is the digestion of cellulose of any economic importance?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 88

SYMBIOTIC NITROGEN FIXATION

The presence of small, tumor-like growths or nodules on the roots of certain plants, especially those of the family Leguminosae, results in an increase in the nitrogen content of the soil.

The formation of nodules on roots is caused by the associated growth of the plant and certain bacterial organisms. The bacteria are members of the genus *Rhizobium* and are commonly referred to as the root-nodule bacteria. The organisms utilize the nitrogen of the atmosphere and synthesize it into nitrogen compounds, which are largely excreted into the soil. The plant obtains its nitrogen from the activities of the organisms, and the bacteria derive their food from the plant. This type of association is called symbiosis.

The organisms are rod-shaped, aerobic, and are commonly present in nodules in irregular shapes. Swollen or vacuolated forms appear to predominate. These bizarre forms are usually referred to as bacteroids. They are usually absent when the organisms are cultivated on artificial media.

Required:

1. Roots of leguminous plants showing the presence of numerous nodules.
2. 2 tubes of Ashby's mannitol phosphate agar.
3. 1 tube of distilled water.
4. 1:1000 aqueous solution of mercuric chloride.
5. 95 per cent alcohol.
6. 5 sterile Petri dishes.
7. Knife.
8. Forceps.
9. Slides.

Procedure:

1. Remove a nodule from the plant root and wash thoroughly in tap water.
2. Transfer the nodule to a Petri dish containing 95 per cent alcohol. Allow the nodule to remain for about ½ min.
3. Remove the nodule from the alcohol and transfer to a second Petri dish containing a 1:1000 aqueous solution of mercuric chloride. Use a pair of sterile forceps.
4. Allow the nodule to remain in the germicidal solution for about 3 min.
5. Transfer the nodule to a dish containing sterile distilled water to wash off most of the mercuric chloride.
6. Melt two tubes of Ashby's mannitol phosphate agar in an Arnold sterilizer or in a pan of boiling water.
7. Allow the agar to cool to about 50°C.
8. Pour the melted and cooled agar into the two Petri dishes.
9. Set the plates aside until the agar has hardened.
10. Carefully flame one side of a slide to destroy any organisms present on the surface.
11. Transfer the nodule to the center of the sterilized slide and crush with the flat side of a previously flamed and cooled knife.
12. Add 1 drop of sterile water to the crushed nodule and mix well.
13. Streak a loopful of the bacterial suspension over the surface of one of the agar plates.
14. Without reinoculating the loop, streak it over the surface of the other agar plate.
15. Invert the plates and incubate at 25°C. for 5 to 10 days.
16. Prepare smears of the nodule suspension and stain with methylene blue and by Gram's method.
17. Examine the slides under the oil-immersion objective.
18. Prepare smears from colonies developing on the plates and stain with methylene blue and by Gram's method.
19. Compare the morphological picture of the cultivated organisms with those from the original nodule.
20. Record your results in the following table:

Organism	Gram stain and morphology
Nodule	
Culture	

Questions:

1. Why is mannitol added to Ashby's medium?
2. Could glucose be substituted for the mannitol?
3. What are bacteroids?
4. Why is the nodule first immersed in alcohol?
5. Why do some species of root-nodule bacteria produce moist mucoid colonies whereas others do not?

6. Are the organisms in culture able to fix nitrogen?
7. What is meant by cross-inoculation groups?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 89

NONSYMBIOTIC NITROGEN FIXATION

The most important organisms capable of fixing nitrogen nonsymbiotically are members of the genus *Azotobacter*. The organisms are very pleomorphic, appearing as large rods, as cocci, and sometimes almost yeast-like.

The organisms may be easily cultivated by inoculating a small amount of fertile soil into an inorganic medium containing mannitol. A heavy pellicle develops on the surface of the medium. A microscopic examination reveals the presence of many typical cells of *Azotobacter* surrounded by slimy capsules. Pure cultures may be obtained by streaking some of the pellicle over the surface of the same medium solidified with agar.

Required:

1. Sample of fertile soil or well-limed and manured field soil.
2. One 500-cc. flask containing 100 cc. of Ashby's mannitol phosphate solution.
3. 2 tubes of Ashby's mannitol phosphate agar.
4. 2 sterile Petri dishes.
5. Slides.

Procedure:

1. Inoculate the flask of Ashby's mannitol phosphate solution with 2 gm. of soil.
2. Incubate the flask at 25°C. until a pellicle is formed on the surface. This requires from 1 to 3 weeks. A large surface is preferable since the organisms are found in large numbers in the pellicle.
3. Do not disturb the pellicle after it has once formed.
4. Prepare a Gram stain from the pellicle and examine the slide for the presence of large typical cells.
5. Describe the morphological picture. Another bacterial species is frequently found growing in the pellicle.
6. Melt two tubes of Ashby's agar in an Arnold sterilizer or in a pan of boiling water.
7. Allow the agar to cool to about 50°C.
8. Pour the melted and cooled agar into two sterile Petri dishes.
9. When the agar has hardened, streak a loopful of the pellicle over the surface of one of the plates.
10. Without reinoculating the wire loop, streak it over the surface of the second plate.
11. Invert the plates and incubate at 25°C. for from 1 to 3 weeks.

12. Prepare Gram stains from characteristic colonies and examine under the oil immersion objective.

13. Record your results in the following table:

Relative size of cells   Pigment   Gram reaction and morphology

Questions:

1. What organism is frequently found associated with *Azotobacter* in the pellicle?
2. Are *Azotobacter* pigmented?
3. Why are cultures of the organism very mucoid?
4. Are laboratory cultures of the organism capable of fixing nitrogen?
5. In what kinds of soils are *Azotobacter* abundantly found?

Reference

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

THE BACTERIAL DISEASES OF PLANTS

EXERCISE 90

STEM ROT OR BLACKLEG OF POTATO

Stem rot or blackleg of potato is one of the most important bacterial diseases of plants. It is caused by the organism *Erwinia phytophthora*. The organism causes a blackening and softening of the shoots at the surface of the earth. The blackening soon spreads to the upper parts. The stems wilt, shrivel, and fall to the ground. The tubers often rot. The disease may be transferred to healthy plants by inoculating young shoots with the organism.

E. phytophthora is a small, Gram-negative, nonspore-forming motile rod.

Required:

1. 24-hr. nutrient broth culture of *Erwinia phytophthora* incubated at 25 to 30°C.
2. Sound, turgid potato.
3. 1 tube of nutrient agar.
4. 1:1000 aqueous solution of mercuric chloride.
5. 95 per cent alcohol.
6. 3 sterile Petri dishes.
7. Two 1000-cc. beakers.
8. Sterile knife.
9. Sterile forceps.
10. Sterile towel.
11. Brush.
12. Slides.

Procedure:

1. Wash the potato thoroughly by scrubbing with a brush.
2. Immerse the potato in 95 per cent alcohol contained in a beaker. This drives out air from the pores and crevices.
3. Transfer the tuber to a solution of 1:1000 mercuric chloride for 30 min. to sterilize the outer surface. Some bacteria are probably not killed but the surface of the potato becomes so impregnated with mercury that the organisms seldom develop or give any trouble.
4. Remove the potato from the mercury solution with sterile forceps and dry between a sterile folded towel.
5. Without removing from the towel cut into slices about ½ in. thick. Use a sterile knife.
6. Transfer a slice to each of two sterile Petri dishes.
7. Inoculate the surface of one of the slices with two loopfuls of a 24-hr. nutrient broth culture of *E. phytophthora*. Keep the other slice for a control.

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8. Incubate the dishes at 25°C. for 48 hr. or until rotting occurs.

9. Remove a loopful of the soft rot and streak it over the surface of a nutrient agar plate.

10. Incubate the plate at 25°C. for 48 hr. If a 25°C. incubator is not available keep the plate in your locker.

11. Prepare a Gram stain from a characteristic colony and compare the morphology with that of the original culture.

12. Record your results in the following table:

Organism	Pigment	Gram reaction and morphology
Culture		
Potato		

Questions:

1. What is tyrosinase?

2. Does cooked potato inoculated with *E. phytophthora* become blackened? Why?

3. Why are moist, turgid potatoes more susceptible to the disease than dry, flabby ones?

4. What tissue of the plant is especially susceptible to attack?

5. Compare the cultural reactions of *E. phytophthora* with those of *E. carotovora*. What do you conclude?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 91

THE CUCURBIT WILT

A number of plants, including cucumber, are susceptible to an infection characterized by wilting of the vines, shriveling, and finally death. It is a widespread vascular disease transmitted by the bite of insects.

The disease first appears on the leaves in the form of dull-green, flabby patches. Later the leaves wilt and shrivel. The organisms pass down into the stem by way of the spiral vessels into the petiole. The bacteria rupture the vessel walls and invade the intercellular spaces of the parenchyma. The cut surfaces of a diseased stem exude a white, viscid liquid heavily infected with bacteria.

The disease is caused by *Erwinia tracheiphila,* a small, grayish-white, viscid, nonspore-forming, Gram-negative, motile rod.

Required:

1. Diseased cucumber stem.
2. 1 tube of nutrient broth.
3. 1 tube of nutrient agar.
4. 1 sterile Petri dish.
5. Knife.
6. Sterile towel.
7. Slides.

Procedure:

1. Wash the diseased stem thoroughly in clean tap water.
2. Select a portion of the stem and sterilize by rolling it a few times over a Bunsen flame. Avoid cooking the interior.
3. Place the flamed stem on a folded sterile towel.
4. Sterilize a knife blade in a Bunsen flame, then cut crosswise through the sterilized portion of the stem.
5. Press down on the stem to squeeze out some of the white, viscid liquid containing organisms.
6. Transfer two loopfuls of this material to a tube of nutrient broth.
7. Incubate the tube at 30°C. for 48 hr.
8. Streak a loopful of the broth culture over the surface of a nutrient agar plate.
9. Incubate the plate at 30°C. for 48 hr.
10. Prepare a Gram stain from a characteristic colony and examine under the oil-immersion objective.
11. Record your results in the following table:

Organism	Pigment	Gram reaction and morphology
Broth		
Agar		

Questions:

1. Do the cultural reactions of the organism differ widely from those of *E. carotovora* and *E. phytophthora?*
2. Can the disease be transferrred to healthy plants by inoculating a culture of the organisms?
3. Does the organism in culture quickly lose its virulence?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 92

CAULIFLOWER SPOT

The disease is characterized by a spotting of the leaves on the veins and in the parenchyma. The spots are first water-soaked, then become brownish to purplish gray by reflected light. Badly spotted leaves may turn yellow, then drop off of the plant. Infection takes place through the stomata. The disease can ·be transmitted to healthy plants by spraying the leaves with a culture of the organism.

The disease is caused by *Phytomonas maculicola*, a small, white, motile, nonsporulating, Gram-negative rod.

Required:

1. Diseased cauliflower or cabbage leaf.
2. 1 tube of nutrient broth.
3. 1 tube of nutrient agar.
4. 100 cc. of sterile distilled water.
5. 1:1000 aqueous solution of mercuric chloride.
6. 95 per cent alcohol.
7. Sterile towel.
8. 3 sterile 400-cc. beakers.
9. 1 sterile Petri dish.
10. Sterile 10-cc. pipette.
11. Sterile mortar and pestle.
12. Sterile forceps.
13. Slides.

Procedure:

1. Wash the diseased leaf in clean tap water.
2. Immerse the leaf in 95 per cent alcohol for about 3 sec. to promote uniform wetting of the surface.
3. With a pair of sterile forceps, transfer the leaf to a beaker containing a 1:1000 aqueous solution of mercuric chloride and allow to remain for ½ min. to destroy surface contaminants.
4. Transfer the leaf to a beaker containing sterile distilled water to remove as much of the disinfectant as possible.
5. Place the leaf in a sterile mortar and crush thoroughly with a pestle.
6. Add about 5 cc. of nutrient broth to the pulp and mix thoroughly.
7. Cover the mortar with a sterile towel and allow to stand for about 1 hr.
8. Streak one loopful of the broth extract over the surface of a nutrient agar plate.
9. Invert the plate and incubate at 25°C. for 48 hr. If a 25°C. incubator is not available, place the plate in your locker.
10. Examine the plate for the presence of whitish, circular, shining, translucent, entire colonies.

11. Prepare a Gram stain from a typical colony and examine under the oil-immersion objective.

12. Record your results in the following table:

Plant material	Pigment	Gram reaction and morphology

Questions:

1. Why should a longer exposure period to mercuric chloride be avoided in sterilizing the outer surface of the leaf?

2. How can the disease be transmitted to healthy plants? .

3. Does the organism lose its virulence readily on laboratory media?

4. How does infection take place in the field?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Bergey's Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

EXERCISE 93

OLIVE TUBERCLE

The development of tumors or galls on various parts of olive trees is the result of infection by the organism *Phytomonas savastanoi*. The attacked parts are dwarfed or killed and occasionally the entire tree is destroyed. A tree seldom recovers from an infection. The organism continues to invade new shoots and different parts of the old tree. The organisms may be readily seen between the cells and intercellular cavities. The surrounding tissue has a water-soaked or brownish appearance. During wet or rainy weather the organisms are secreted from the tumors and washed to other parts of the same tree and probably to other trees. Tree wounds offer a portal of entry to the organisms, resulting in new infections.

The organism is a small, white, motile, nonspore-forming, Gram-negative rod.

Required:

1. Olive tumor.
2. 1 tube of nutrient broth.
3. 1 tube of nutrient agar.
4. 100 cc. of sterile distilled water.
5. 95 per cent alcohol.
6. 1:1000 aqueous solution of mercuric chloride.

7. Sterile Petri dish.
8. Sterile mortar and pestle.
9. 3 sterile 400-cc. beakers.
10. Sterile 10-cc. pipette.
11. Sterile towel.
12. Sterile forceps.
13. Knife.
14. Slides.

Procedure:

1. Wash the tumor thoroughly in clean tap water.
2. Scrape the tumor with a knife.
3. Immerse the specimen in 95 per cent alcohol for about 10 sec. to promote uniform wetting of the surface.
4. With a pair of sterile forceps, transfer the tumor to a beaker containing a 1:1000 aqueous solution of mercuric chloride and allow to remain for 5 min. to destroy surface contaminants.
5. Transfer the tumor to a beaker containing sterile distilled water to remove as much of the disinfectant as possible.
6. Place the tumor in a sterile mortar and crush thoroughly with a pestle.
7. Add about 5 cc. of nutrient broth to the pulp and mix thoroughly.
8. Cover the mortar with a sterile towel and allow to stand for about 1 hr.
9. Streak one loopful of the broth extract over the surface of a nutrient agar plate.
10. Invert the plate and incubate at 25°C. for 48 hr. If a 25°C. incubator is not available, place the plate in your locker.
11. Examine the plate for the presence of white, smooth, flat, glistening, entire colonies.
12. Prepare a Gram stain from a typical colony and examine under the oil-immersion objective.
13. Record your results in the following table:

Plant material	Pigment	Gram reaction and morphology

Questions:

1. What is the origin of the term *savastanoi?*
2. What is the origin of the term *Phytomonas?*
3. Do the organisms appear intercellular or intracellular in the tubercles?
4. Are all species of olive trees equally susceptible to the disease?

References

BERGEY, D. H., R. S. BREED, E. G. D. MURRAY, and A. P. HITCHENS: "Le....y. Manual of Determinative Bacteriology," Baltimore, The Williams & Wilkins Company, 1939.

SALLE, A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

INFECTION AND IMMUNITY

THE AGGLUTINATION OF BACTERIA

When a bacterial suspension is mixed with specific antiserum (previously heated to 56°C. for ½ hr.), a gathering together or clumping of the organisms occurs. This is due to the presence in the immune serum of antibodies known as agglutinins. The organisms (antigen) are spoken of as agglutinogens.

Agglutination does not occur in the complete absence of electrolyte. However, the addition of only a minute amount of electrolyte to a nonagglutinating mixture of antigen and antibody causes agglutination to take place. This applies to other cells as well as to bacteria. Agglutination is believed to proceed in two stages: (1) the antibody becomes fixed to the antigen and (2) the cells clump together in the presence of an electrolyte.

The test is of great value in certain diagnostic procedures.

Required:

1. *Escherichia coli* immune serum (1:20).
2. 24-hr. nutrient agar slant culture of *E. coli*.
3. 1 tube of 0.85 per cent NaCl solution.
4. 7 small test tubes or agglutination tubes.
5. 1 test-tube rack.
6. 1 sterile test tube.
7. 2 sterile 1-cc. pipettes.
8. Sterile 10-cc. pipette.

Procedure:

1. Prepare a suspension of *E. coli* by adding 5 cc. of 0.85 per cent NaCl solution to a 24-hr. nutrient agar slant culture of the organism.

2. Suspend the organisms in the salt solution by rotating the tube between the palms of the hands.

3. Allow to stand a few minutes for the coarse particles to settle out.

4. Transfer 5 cc. of 0.85 per cent NaCl solution to a sterile test tube (⅝ by 6 in.).

5. With the same pipette transfer 2 cc. of the bacterial suspension to the same tube.

6. The suspension should be distinctly turbid. The degree of turbidity may be determined by holding an inoculating needle between the suspension and an electric lamp. If the wire is just visible when it is placed 1 in. from the bacterial suspension, the degree of turbidity is approximately correct.

7. Arrange seven small test tubes or agglutination tubes in a rack and number from 1 to 7.

8. Pipette into each tube 0.5 cc. of 0.85 per cent NaCl solution.

9. Add 0.5 cc. of *E. coli* antiserum (1:20) to tube 1.

10. Mix by drawing the contents into a pipette and expelling. Repeat this 2 or 3 times.

11. Remove 0.5 cc. from tube 1 and transfer to tube 2.

12. Mix as before.

13. Continue with this same operation through tube 6.

14. Withdraw 0.5 cc. of the mixture from tube 6 and discard it. Do not transfer any antiserum to tube 7. This serves as a check on spontaneous agglutination.

15. Each tube should now contain 0.5 cc. of liquid.

16. Add to each tube 0.5 cc. of the *E. coli* suspension, and shake well to obtain a uniform mixture.

17. Incubate the tubes in a water bath at 55°C. for 1 hr.

18. Examine the tubes for the presence of agglutination.

19. Place the tubes in the refrigerator over night and again examine for agglutination.

20. Record your results in the following table:

DILUTION OF *E. coli* ANTISERUM

1:40	1:80	1:160	1:320	1:640	1:1280	Control

21. Record agglutination as $+++$, $++$, $+$, 0, depending upon the degree of clumping.

Questions:

1. What is an antigen?
2. Why is the antiserum heated to 56°C. for ½ hr. previous to use?
3. Is agglutination a two-component or a three-component system?
4. Why is an electrolyte necessary for agglutination to occur?
5. Could heat-killed bacteria be used as the antigen?
6. Define the term *titer*.
7. What is meant by the *zone phenomenon?*

Reference

SALLE A. J.: "Fundamental Principles of Bacteriology," 2d ed., New York, McGraw-Hill Book Company, Inc., 1942.

APPENDIX

PREPARATION OF SOLUTIONS AND STAINS

The preparations are arranged in alphabetical order.

1. *Acetone Alcohol*

Alcohol (95 per cent).. 700 cc.
Acetone.. 300 cc.
Mix them.

2. *Acid Alcohol*

Hydrochloric acid (37 per cent) c.p...................... 30 cc.
Alcohol (95 per cent), sufficient to make 1000 cc.
Dissolve the hydrochloric acid in sufficient alcohol to make 1000 cc.

3. *Albert's Diphtheria Stain*

Toluidine blue.. 0.15 gm.
Acetic acid (glacial) c.p................................. 1 cc.
Alcohol (95 per cent)..................................... 2 cc.
Distilled water... 100 cc.
Dissolve the toluidine blue in the distilled water. Then add the acetic acid and alcohol and mix well.

4 *α-Naphthylamine Test Solution*

α-Naphthylamine.. 5 gm.
Sulfuric acid (conc.) c.p................................. 8 cc.
Distilled water... 1000 cc.
Add the sulfuric acid to the distilled water and mix well. Then add the α-naphthylamine and stir until dissolved.

5. *Boric Acid 0.2M Solution*

Boric acid c.p. crystals.................................. 12.4 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the boric acid in sufficient distilled water to make 1000 cc.

6. *Bromocresol Green Indicator Solution*

Bromocresol green.. 0.4 gm.
Alcohol (95 per cent)..................................... 500 cc.
Distilled water... 500 cc.
Dissolve the bromocresol green in the alcohol. Add the distilled water and filter the solution through paper.

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7. Bromocresol Purple Indicator Solution for Fermentation Media

Bromocresol purple.. 16 gm.
Alcohol (95 per cent)...................................... 500 cc.
Distilled water.. 500 cc.

Dissolve the bromocresol purple in the alcohol. Add the distilled water and filter the solution through paper.

8. Bromothymol Blue Indicator Solution

Bromothymol blue.. 0.4 gm.
Alcohol (95 per cent)...................................... 500 cc.
Distilled water.. 500 cc.

Dissolve the bromothymol blue in the alcohol. Add the distilled water and filter the solution through paper.

9. Bromothymol Blue Indicator Solution for Fermentation Media†

Bromothymol blue.. 16 gm.
Alcohol (95 per cent)...................................... 500 cc.
Distilled water.. 500 cc.

Dissolve the bromothymol blue in the alcohol. Add the distilled water and filter the solution through paper.

10. Citric Acid 0.1M Solution

Citric acid c.p.. 21 gm.
Distilled water, sufficient to make 1000 cc.

Dissolve the citric acid in sufficient distilled water to make 1000 cc.

11. Cleaning Solution

Sodium dichromate......................... • .... 25 gm.
Sulfuric acid (conc. tech) 1000 cc.

Dissolve the sodium dichromate in 50 cc. of water and add slowly, with constant stirring, to 1000 cc. of sulfuric acid.

12. Compound Solution of Cresol (5 per cent)

Compound solution of cresol (U.S.P.)‡..... 100 cc.
Distilled water, sufficient to make 1000 cc.

Mix them.

13. Copper Sulfate 20 Per Cent Solution

Copper sulfate ($CuSO_4 \cdot 5H_2O$) c.p...................... 200 gm.
Distilled water, sufficient to make 1000 cc.

Dissolve the copper sulfate in sufficient distilled water to make 1000 cc.

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* This solution is to be used for the preparation of bromocresol purple milk and lactose bromocresol purple agar. It is not intended for use as an indicator for adjusting the reaction of culture media, a 0.04 per cent solution being used for that purpose.

† This solution is to be used for the preparation of fermentation media. It is not intended for use as an indicator for adjusting the reaction of culture media, a 0.04 per cent solution being used for that purpose.

‡ Compound solution of cresol (U.S.P.) is a 50 per cent solution of cresol.

14. *Crystal Violet* 1 *Per Cent Solution*

Crystal violet (90 per cent dye content).................. 10 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the crystal violet in sufficient distilled water to make 1000 cc.

15. *Crystal Violet* 1:1000 *Solution*

Crystal Violet (90 per cent dye content).................. 1 gm.
Distilled water...................................... 1000 cc.
Dissolve the crystal violet in the distilled water.

16. *Crystal Violet* 1:5000 *Solution*

Crystal violet (90 per cent dye content)................. 0.2 gm.
Distilled water...................................... 1000 cc.
Dissolve the crystal violet in the distilled water.

17. *Diphenylamine Reagent*

Diphenylamine c.p.................................... 0.5 gm.
Distilled water...................................... 20 cc.
Sulfuric acid (conc.)................................. 100 cc.
Add the sulfuric acid slowly to the distilled water with constant stirring. Then add the diphenylamine and stir until dissolved.

18. *Ehrlich-Böhme Test Solution*

Solution A.
Paradimethylaminobenzaldehyde .... 1 gm.
Alcohol (95 per cent)................................. 95 cc.
Hydrochloric acid (conc.)............................. 20 cc.
Dissolve the paradimethylaminobenzaldehyde in the alcohol. Then add the hydrochloric acid and mix well.
Solution B.
Saturated aqueous solution of potassium persulfate ($K_2S_2O_8$).

19. *Ferric Chloride* 2 *Per Cent Solution*

Ferric chloride ($FeCl_3 \cdot 6H_2O$) c.p....................... 2 gm.
Distilled water, sufficient to make 100 cc.
Dissolve the ferric chloride in sufficient distilled water to make 100 cc.

20. *Gnezda Oxalic Acid Test Papers*

Dip a piece of filter paper in a warm saturated aqueous solution of oxalic acid. Hang it up to dry. On cooling, the paper is covered with crystals of oxalic acid. When thoroughly dry, cut the paper into strips of approximately ¼ by 3 in. and preserve in a corked wide-mouth bottle.

21. *Gram's Iodine Solution*

Iodine, c.p... 1 gm.
Potassium iodide c.p.................................. 2 gm.
Distilled water...................................... 300 cc.
Mix the iodine and potassium iodide in a mortar and triturate with a pestle until finely divided. Add distilled water in small portions to wash out the contents into a graduate. Finally add sufficient distilled water to make 300 cc. Mix well.

22. *Gray's Mordant*

Solution A.

Aluminum and potassium sulfate [AlK$(SO_4)_2 \cdot 12H_2O$] c.p. saturated aqueous solution.. 5 cc.
Tannic acid c.p. (20 per cent aqueous sol.)..................... 2 cc.
Mercuric chloride c.p. (sat. aqueous sol.)..................... 2 cc.
Solution B.
Basic fuchsin (sat. alcoholic sol.)........................... 0.4 cc.

Mix solutions A and B within 24 hr. before using. The solutions keep indefinitely in separate containers but deteriorate rapidly when mixed.

23. *Hiss' Capsule Stain*

Crystal violet (90 per cent dye content), sat. alcoholic sol..... 10 cc.
Distilled water, sufficient to make 100 cc.

Mix the crystal violet solution with sufficient distilled water to make 100 cc.

24. *Hucker's Ammonium Oxalate Crystal Violet Stain*

Solution A.
Crystal violet (90 per cent dye content)................... 3 gm.
Alcohol (95 per cent)................................... 20 cc.
Solution B.
Ammonium oxalate c.p................................ 0.8 gm.
Distilled water.. 80 cc.

Mix solutions A and B.

25. *Hydrochloric Acid 0.1N Solution**

Hydrochloric acid (37 per cent) c.p....................... 10 cc.
Distilled water, sufficient to make 1000 cc.

Dissolve the hydrochloric acid in sufficient distilled water to make 1000 cc.

26. *Lactophenol Solution*

Phenol, c.p. crystals................................... 20 gm.
Lactic acid, c.p....................................... 20 cc.
Glycerin, c.p.. 40 cc.
Distilled water.. 40 cc.

Mix the ingredients and preserve in a brown bottle to keep the solution in the colorless form.

27. *Lugol's Iodine Solution*

Iodine, c.p.. 50 gm.
Potassium iodide c.p................................... 100 gm.
Distilled water, sufficient to make 1000 cc.

Mix the iodine and potassium iodide in a mortar and triturate with a pestle until finely divided. Add distilled water in small portions to wash out the contents into a graduate. Finally add sufficient distilled water to make 1000 cc. Mix well.

28. *Malachite Green 5 Per Cent Solution*

Malachite green (90 per cent dye content)................... 5 gm.
Distilled water, sufficient to make 100 cc.

Dissolve the malachite green in the distilled water.

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* The strength of this solution is only approximate, but sufficiently accurate for use in the experimental procedures in this manual.

29. *Mercuric Chloride* 1:1000 *Solution*

    Mercuric chloride c.p.................................. 1 gm.
    Distilled water...................................... 1000 cc.
Dissolve the mercuric chloride in 1000 cc. of distilled water.

30. *Methyl Red Indicator Solution*

    Methyl red... 0.1 gm.
    Alcohol (95 per cent).............................. 250 cc.
    Distilled water.................................... 250 cc.
Dissolve the methyl red in the alcohol. Add the distilled water and filter through paper.

31. *Methylene Blue* 1:20,000 *Solution*

    Methylene blue (90 per cent dye content).............. 0.05 gm.
    Distilled water.................................... 1000 cc.
Dissolve the methylene blue in the distilled water.

32. *Methylene Blue Staining Solution*

    Methylene blue (90 per cent dye content).............. 0.3 gm.
    Alcohol (95 per cent).............................. 30 cc.
    Distilled water.................................... 100 cc.
Dissolve the methylene blue in the alcohol. Add the distilled water and filter the solution through paper.

33. *Neisser's Diphtheria Stain*

    Solution 1.
    Methylene blue (90 per cent dye content)................. 1 gm.
    Alcohol (95 per cent).............................. 20 cc.
    Acetic acid glacial c.p............................ 50 cc.
    Distilled water.................................... 950 cc.
Dissolve the methylene blue in the alcohol. Add the other ingredients and filter the solution through paper.
    Solution 2.
    Crystal violet (90 per cent dye content)................. 1 gm.
    Alcohol (95 per cent)............................. 10 cc.
    Distilled water.................................... 300 cc.
Dissolve the crystal violet in the alcohol. Add the distilled water and filter the solution through paper.
    Solution 3.
    Chrysoidin... 2 gm.
    Hot distilled water................................ 300 cc.
Filter the solution through paper.

34. *Nitric Acid* 10 *Per Cent Solution*

    Nitric acid (69 per cent) c.p....................... 145 cc.
    Distilled water, sufficient to make 1000 cc.
Mix them.

35. *Phenol 5 Per Cent Solution*

    Phenol c.p. crystals............................... 50 gm.
    Distilled water, sufficient to make 1000 cc.
Dissolve the phenol in sufficient distilled water to make 1000 cc.

36. *Phenol Red Indicator Solution*

Phenol red... 0.2 gm.
Alcohol (95 per cent)................................ 500 cc.
Distilled water...................................... 500 cc.
Dissolve the phenol red in the alcohol.   Add the distilled water and filter the solution through paper.

37. *Plaster of Paris Blocks*

Plaster of paris ($CaSO_4 \cdot 2H_2O$)............................ 2 parts
Distilled water...................................... 1 part
Mix the calcium sulfate and the distilled water and mold into small blocks 1 by 1 by ⅜ in.

38. *Plimmer and Paine's Mordant*

Tannic acid c.p...................................... 10 gm.
Aluminum chloride ($AlCl_3 \cdot 6H_2O$) c.p.................... 18 gm.
Zinc chloride c.p.................................... 10 gm.
Basic fuchsin (90 per cent dye content)................. 1.5 gm.
Alcohol (60 per cent)................................ 40 cc.
Triturate the solids in a mortar with about 10 cc. of alcohol.   Then slowly add the remainder of the alcohol, triturating after each addition until solution is effected. This solution may be kept for years.

39. *Potassium Phosphate (Secondary) 0.2M Solution*

Potassium phosphate (K_2HPO_4) c.p................... 34.9 gm.
Distilled water, sufficient to make..................... 1000 cc.
Dissolve the sodium phosphate in sufficient distilled water to make 1000 cc.

40. *Rose Bengal 1 Per Cent Solution*

Rose bengal (80 per cent dye content)................. 1 gm.
Calcium chloride ($CaCl_2 \cdot 2H_2O$) c.p.................... 0.01 gm.
Phenol c.p.. 5 gm.
Distilled water...................................... 100 cc.
Dissolve the phenol in the distilled water.   Add the other ingredients and stir until dissolved.   Filter the solution through paper.

41. *Safranine Staining Solution*

Safranine (90 per cent dye content)..................... 0.25 gm.
Alcohol (95 per cent)................................ 10 cc.
Distilled water...................................... 100 cc.
Dissolve the safranine in the alcohol.   Add the distilled water and filter the solution through paper.

42. *Safranine 0.5 Per Cent Solution*

Safranine (90 per cent dye content)..................... 0.5 gm.
Distilled water, sufficient to make 100 cc.
Dissolve the safranine in sufficient distilled water to make 100 cc.

43. *Saturated Aqueous or Alcoholic Solutions*

These are prepared by adding an excess of the solute to the solvent and shaking until the solution becomes saturated.

44. *Sodium Chloride 0.85 Per Cent Solution*

Sodium chloride c.p.. 8.5 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the sodium chloride in sufficient distilled water to make 1000 cc.

45. *Sodium Chloride 30 Per Cent Solution*

Sodium chloride c.p.. 300 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the sodium chloride in sufficient distilled water to make 1000 cc.

46. *Sodium Hydroxide 0.2M Solution**

Sodium hydroxide c.p... 9 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the sodium hydroxide in sufficient distilled water to make 1000 cc.
Preserve in rubber-stoppered bottles.

47. *Sodium Hydroxide 1N Solution†*

Sodium hydroxide c.p... 40 gm.
Distilled water sufficient to make 1000 cc.
Dissolve the sodium hydroxide in sufficient distilled water to make 1000 cc.
Preserve in rubber-stoppered bottles.

48. *Sodium Hydroxide 10 Per Cent Solution*

Sodium hydroxide c.p... 100 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the sodium hydroxide in sufficient distilled water to make 1000 cc.
Preserve in rubber-stoppered bottles.

49. *Sodium Hydroxide 0.05N Solution*

Sodium hydroxide 1N solution.......................... 50 cc.
Distilled water, sufficient to make 1000 cc.
Add sufficient distilled water to the 1N solution of sodium hydroxide to make 1000 cc. Preserve in a rubber-stoppered bottle.

50. *Sodium Phosphate (Primary) 1 Per Cent Solution*

Sodium phosphate ($NaH_2PO_4 \cdot H_2O$) c.p.................... 10 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the primary sodium phosphate in sufficient distilled water to make 1000 cc.

51. *Sodium Phosphate (Secondary) 1 Per Cent Solution*

Sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) c.p................. 10 gm.
Distilled water, sufficient to make 1000 cc.
Dissolve the secondary sodium phosphate in sufficient distilled water to make 1000 cc.

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* This is only approximate since the sodium hydroxide is not 100 per cent pure and the sticks quickly absorb water on exposure to air. However, a solution of approximate strength is sufficiently accurate for use in the experimental procedures in this manual.

† The same remarks apply here as given above.

52. *Sodium Ricinoleate* 1 *Per Cent Solution*

Sodium ricinoleate...................................... 1 gm.
Distilled water, sufficient to make 100 cc.

Dissolve the sodium ricinoleate in sufficient distilled water to make 100 cc. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

53. *Sucrose* 40 *Per Cent Solution*

Sucrose (cane sugar)................................... 400 gm.
Distilled water, sufficient to make 1000 cc.

Dissolve the sugar in sufficient distilled water to make 1000 cc.

54. *Sulfanilic Acid Test Solution*

Sulfanilic acid c.p.................................... 8 gm.
Sulfuric acid (conc.) c.p.............................. 48 cc.
Distilled water, sufficient to make 1000 cc.

Add the sulfuric acid to 500 cc. of distilled water. Then add the sulfanilic acid and finally sufficient distilled water to make 1000 cc.

55. *Vaspar*

Vaseline... 1 part
Paraffin... 1 part

Heat the two together until melted. Mix well and pour into test tubes.

56. *Ziehl-Neelsen's Carbolfuchsin Stain*

Basic fuchsin (90 per cent dye content).................. 0.3 gm.
Alcohol (95 per cent).................................. 10 cc.
Phenol c.p. crystals................................... 5 gm.
Distilled water.. 95 cc.

Dissolve the basic fuchsin in the alcohol. Dissolve the phenol in the distilled water. Mix the two solutions.

PREPARATION OF CULTURE MEDIA

The media are arranged in alphabetical order.

1. *Ashby's Mannitol Phosphate Agar*

Potassium acid phosphate (KH_2PO_4) c.p................	0.2 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p................	0.2 gm.
Mannitol...	10.0 gm.
Sodium chloride c.p................................	0.2 gm.
Calcium sulfate ($CaSO_4 \cdot 2H_2O$) c.p..................	0.1 gm.
Calcium carbonate c.p.............................	5 gm.
Agar...	20.0 gm.
Distilled water...................................	1000 cc.

Dissolve the phosphate in 500 cc. of distilled water; then add $1N$ NaOH until the solution is neutral to phenolphthalein. Add 500 cc. more of distilled water and the other ingredients in the order named. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Do not filter. Dispense into test tubes. Stir often while tubing to keep the $CaCO_3$ in suspension. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

2. *Ashby's Mannitol Phosphate Solution*

Potassium acid phosphate (KH_2PO_4) c.p................	0.2 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p................	0.2 gm.
Mannitol...	10.0 gm.
Sodium chloride c.p................................	0.2 gm.
Calcium sulfate ($CaSO_4 \cdot 2H_2O$) c.p..................	0.1 gm.
Calcium carbonate c.p.............................	5.0 gm.
Distilled water...................................	1000 cc.

Dissolve the phosphate in 500 cc. of distilled water; then add $1N$ NaOH until the solution is neutral to phenolphthalein. Add 500 cc. more of distilled water and the other ingredients in the order named. Do not filter. Dispense into flasks. Stir often to keep the $CaCO_3$ in suspension. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

3. *Beijerinck's Thiosulfate Agar*

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) c.p................	5.0 gm.
Dipotassium phosphate (K_2HPO_4) c.p................	0.1 gm.
Sodium bicarbonate c.p............................	0.2 gm.
Ammonium chloride c.p.............................	0.1 gm.
Agar..	20 gm.
Distilled water...................................	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

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4. *Beijerinck's Thiosulfate Solution*

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) c.p.................	5.0 gm.
Ammonium chloride c.p..............................	0.1 gm.
Sodium bicarbonate c.p.............................	1.0 gm.
Disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) c.p.............	0.2 gm.
Magnesium chloride ($MgCl_2 \cdot 6H_2O$) c.p.................	0.1 gm.
Ferrous sulfate ($FeSO_3 \cdot 7H_2O$)........................	Trace
Distilled water....................................	1000 cc.

Dissolve the salts in the distilled water in the order named. Dispense into flasks. Sterilization is not necessary.

5. *Bromocresol Purple Milk*

Fresh milk (skimmed)..................................	1000 cc.
Bromocresol purple indicator solution (Solution 7, page 168)..	1 cc.

Add the bromocresol purple indicator solution to the skimmed milk and mix well. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

6. *Cobalt-Nickel Agar Medium*

Proteose peptone (Difco)................................	20 gm.
Dipotassium phosphate (K_2HPO_4) c.p....................	1 gm.
Glucose...	1 gm.
Cysteine hydrochloride................................	2 gm.
Agar..	15 gm.
0.005M cobalt nitrate solution*........................	20 cc.
0.005M nickel nitrate solution†........................	100 cc.
Distilled water.......................................	880 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Pour the medium into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

7. *Dextrin Fermentation Broth*

Meat extract..	3 gm.
Peptone..	5 gm.
Dextrin..	5 gm.
Bromothymol blue indicator solution (Solution 9, page 168).	1 cc.
Distilled water......................................	1000 cc.

Mix the meat extract, peptone, and dextrin with the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Add the indicator solution and mix thoroughly. Filter through paper. Dispense into fermentation tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

8. *Endo Agar*

Peptone..	10 gm.
Dipotassium phosphate (K_2HPO_4) c.p.................	3.5 gm.
Agar...	20 gm.
Distilled water......................................	1000 cc.

* 0.727 gm. $CO(NO_3)_2 \cdot 6H_2O$ c.p. in 500 cc. distilled water.
† 0.728 gm. $Ni(NO_3)_2 \cdot 6H_2O$ c.p. in 500 cc. distilled water.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. No adjustment of the reaction is necessary. Dispense in 100-cc. amounts into flasks. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

When ready to use, melt a flask of agar (100 cc.) in an Arnold and add the following:

    a. Lactose.. 1 gm.

    b. A freshly prepared solution consisting of 0.5 gm. of sodium
        sulfite (anhydrous) and 2 cc. of basic fuchsin (10 per cent
        alcoholic solution) dissolved in 5 cc. of distilled water.

Mix the contents of the flask thoroughly. Steam in an Arnold for 5 min. Allow the agar to cool to about 50°C.; then pour into Petri dishes (about eight plates).

9. *Eosin Methylene Blue Agar*

Peptone... 10 gm.
Dipotassium phosphate (K_2HPO_4)...................... 2 gm.
Agar... 20 gm.
Distilled water....................................... 1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. No adjustment of the reaction is necessary. Dispense in 100-cc. amounts into flasks. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

When ready to use, melt a flask of agar (100 cc.) in an Arnold and add the following:

Lactose.. 1 gm.
Eosin (2 per cent solution)............................ 2 cc.
Methylene blue (0.5 per cent solution)................... 1.25 cc.

Mix the contents of the flask thoroughly. Steam in an Arnold for 5 min. Allow the agar to cool to about 50°C.; then pour into Petri dishes (about eight plates).

10. *Glucose Agar*

Meat extract... 3 gm.
Peptone... 5 gm.
Glucose... 10 gm.
Agar.. 20 gm.
Distilled water....................................... 1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Filter through cotton. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

11. *Glucose Blood Agar*

Glucose agar... 100 cc.
Blood (rabbit, guinea pig, horse, human)................. 5 cc.

Melt a 100-cc. flask of glucose agar in an Arnold sterilizer. Allow the agar to cool to a temperature of 50°C. Add the blood and mix thoroughly. Pour into Petri dishes (about eight plates).

12. *Glucose Brain Medium*

Boil sheep brains with an equal volume of distilled water. Decant the water (save) and press the brains through a ricer.

Mix the decanted water with the minced brains and for every 1000 cc. of the suspension add:

Glucose... 1 gm.
Peptone... 20 gm.

Dispense into test tubes in deep layers. Stir often while tubing to keep the minced brains in suspension. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

13. *Glucose Broth*

Meat extract... 3 gm.
Peptone... 5 gm.
Glucose... 5 gm.
Distilled water....................................... 1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.2. Filter through paper. Dispense into tubes or flasks. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

14. *Glucose Fermentation Broth*

Meat extract... 3 gm.
Peptone... 5 gm.
Glucose... 5 gm.
Bromothymol blue indicator solution (Solution 9, page 168). 1 cc.
Distilled water....................................... 1000 cc.

Mix the meat extract, peptone, and glucose with the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Add the indicator solution and mix thoroughly. Filter through paper. Dispense into fermentation tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

15. *Glucose Yeast Extract Agar*

Yeast extract.. 5 gm.
Peptone... 5 gm.
Glucose... 10 gm.
Agar.. 20 gm.
Distilled water....................................... 1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Filter through cotton. Dispense into tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

16. *Kappen's Urea Solution*

Meat extract... 10 gm.
Urea.. 50 gm.
Distilled water....................................... 1000 cc.

Dissolve the ingredients in the distilled water. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

17. *Koser's Citrate Medium*

Sodium ammonium phosphate c.p...................... 1.5 gm.
Potassium acid phosphate (KH_2PO_4) c.p............. 1 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p.......... 0.2 gm.
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) c.p...... 3 gm.
Distilled water....................................... 1000 cc.

Dissolve the salts in the distilled water. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

18. *Koser's Uric Acid Medium*

Sodium chloride c.p.	5 gm.
Magnesium sulfate (MgSO₄·7H₂O) c.p.	0.2 gm.
Calcium chloride (CaCl₂·2H₂O) c.p.	0.1 gm.
Dipotassium phosphate (K₂HPO₄) c.p.	1 gm.
Glycerol c.p.	30.1 gm.
Uric acid	0.5 gm.
Distilled water	1000 cc.

Dissolve the ingredients in the distilled water. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

19. *Lactose Fermentation Broth*

Meat extract	3 gm.
Peptone	5 gm.
Lactose	5 gm.
Bromothymol blue indicator solution (Solution 9, page 168).	1 cc.
Distilled water	1000 cc.

Mix the meat extract, peptone, and lactose with the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Add the indicator solution and mix thoroughly. Filter through paper. Dispense into fermentation tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

20. *Lactose Bromocresol Purple Agar*

Meat extract	3 gm.
Peptone	5 gm.
Lactose	10 gm.
Bromocresol purple indicator solution (Solution 7, page 168)	1 cc.
Agar	20 gm.
Distilled water	1000 cc.

Mix the meat extract, peptone, lactose, and agar with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Add the indicator solution and mix thoroughly. Filter through cotton. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

21. *Lactose Litmus Agar*

Meat extract	3 gm.
Peptone	5 gm.
Lactose	10 gm.
Azolitmin	1 gm.
Agar	20 gm.
Distilled water	1000 cc.

Dissolve the azolitmin in about 10 cc. of distilled water with the aid of a few drops of 1N NaOH.

Mix the remaining ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Add the indicator solution and mix thoroughly. Filter through cotton. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

22. *Litmus Milk*

Fresh milk (skimmed)................................... 1000 cc.
Azolitmin... 1 gm.

Dissolve the azolitmin in about 10 cc. of distilled water with the aid of $1N$ NaOH. Add the indicator solution to the skimmed milk and mix thoroughly. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

23. *Loeffler's Blood Serum Medium*

Serum (beef)... 300 cc.
Glucose broth (medium 13)............................. 100 cc.

Mix the ingredients and dispense 5-cc. amounts into test tubes. Place the tubes in a slanted position in the autoclave. Close the door and all valves before turning on the steam. Run the pressure to 15 lb. as quickly as possible and hold it for 10 min. Do not allow the pressure to go above 15 lb.

When coagulation is complete open the lower valve to replace the trapped air with steam. Sterilize at 15 lb. for 20 min.

24. *Mannose Fermentation Broth*

Meat extract.. 3 gm.
Peptone... 5 gm.
Mannose... 5 gm.
Bromothymol blue indicator solution (Solution 9, page 168). 1 cc.
Distilled water....................................... 1000 cc.

Mix the meat extract, peptone, and mannose with the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Add the indicator solution and mix thoroughly. Filter through paper. Dispense into fermentation tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

25. *McBeth's Cellulose Ammonium Sulfate Solution*

Dipotassium phosphate (K_2HPO_4) c.p.................. 1 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p............ 1 gm.
Sodium carbonate (Na_2CO_3) c.p...................... 1 gm.
Ammonium sulfate c.p.................................. 2 gm.
Calcium carbonate c.p................................. 2 gm.
Distilled water....................................... 1000 cc.
Strip of filter paper.

Mix the ingredients with the distilled water. Dispense into test tubes containing strip of filter paper (cellulose). Stir the solution often while tubing to keep the calcium carbonate in suspension. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

26. *Methyl Red Broth*

Proteose peptone...................................... 7 gm.
Dipotassium phosphate (K_2HPO_4) c.p................. 5 gm.
Glucose... 5 gm.
Distilled water....................................... 1000 cc.

Mix the ingredients with the distilled water. Bring the solution to a boil. Filter through paper. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

27. *Nitrate Broth*

Meat extract	3 gm.
Peptone	5 gm.
Potassium nitrate c.p.	1 gm.
Distilled water	1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.2. Filter through paper. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

28. *Nutrient Agar*

Meat extract	3 gm.
Peptone	5 gm.
Agar	20 gm.
Distilled water	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2. Filter through cotton. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure. This is used for slant cultures and for agar deeps.

29. *Nutrient Broth*

Meat extract	3 gm.
Peptone	5 gm.
Distilled water	1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.2. Filter through paper. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

30. *Nutrient Gelatin*

Meat extract	3 gm.
Peptone	5 gm.
Gelatin	150 gm.
Distilled water	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil. Adjust the reaction to pH7.2. Filter through cotton. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

31. *Sodium Monochloroacetate Agar*

Meat extract	3 gm.
Peptone	5 gm.
Monochloroacetic acid	2 gm.
Agar	20 gm.
Distilled water	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.2 with sodium hydroxide. Filter through cotton. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

32. *Sodium Nitrite Medium*

Sodium nitrite c.p....................................	1.0 gm.
Sodium carbonate (Na_2CO_3) c.p......................	1.0 gm.
Dipotassium phosphate (K_2HPO_4) c.p..................	0.5 gm.
Sodium chloride c.p....................................	0.5 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p.................	0.3 gm.
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) c.p....................	Trace
Distilled water.......................................	1000 cc.

Dissolve the salts in the distilled water in the order named. No adjustment of the reaction is necessary. Dispense into flasks. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

33. *Starch Agar*

Meat extract...	3 gm.
Soluble starch.......................................	10 gm.
Agar...	20 gm.
Distilled water......................................	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Adjust the reaction to pH7.4. Filter through cotton. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

34. *Starch Broth*

Meat extract...	3 gm.
Soluble starch.......................................	10 gm.
Distilled water......................................	1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.4. Filter through cotton. Dispense into test tubes or flasks. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

35. *Sterile Milk*

Fresh milk (skimmed).
Dispense the skimmed milk into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

36. *Sucrose Broth*

Meat extract...	3 gm.
Peptone..	5 gm.
Sucrose..	5 gm.
Distilled water......................................	1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Filter through paper. Dispense into test tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

37. *Sucrose Fermentation Broth*

Meat extract...	3 gm.
Peptone..	5 gm.
Sucrose..	5 gm.
Bromothymol blue indicator solution (Solution 9, page 168).	1 cc.
Distilled water......................................	1000 cc.

Mix the meat extract, peptone, and sucrose with the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.0. Add the indicator solution and mix thoroughly. Filter through paper. Dispense into fermentation tubes. Sterilize in an Arnold for 20 min. at 100°C. on three successive days.

38. *Thioglycollate Broth**

Pork infusion solids (Infusion from 375 gm. pork)......	10 cc.
Peptone..	10 gm.
Sodium chloride.................................	5 gm.
Sodium thioglycollate............................	1 gm.
Agar...	0.5 gm.
Methylene blue.................................	0.002 gm.

Distilled water, sufficient to make 1000 cc.

39. *Tryptone Broth*

Tryptone..	10 gm.
Meat extract......................................	3 gm.
Distilled water...................................	1000 cc.

Dissolve the ingredients in the distilled water. Bring the solution to a boil. Adjust the reaction to pH7.2. Filter through paper. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

40. *Waksman's Sodium Thiosulfate Agar*

Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) c.p...............	5.0 gm.
Ammonium chloride c.p............................	0.1 gm.
Calcium chloride ($CaCl_2 \cdot 6H_2O$) c.p..................	0.25 gm.
Magnesium chloride ($MgCl_2 \cdot 6H_2O$) c.p...............	0.1 gm.
Potassium acid phosphate (KH_2PO_4) c.p..............	3.0 gm.
Agar...	20 gm.
Distilled water...................................	1000 cc.

Mix the ingredients with the distilled water. Bring to a boil and continue to heat until the agar is dissolved. Make up the loss due to evaporation. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

41. *Waksman and Lomanitz's Synthetic Medium*

Dipotassium phosphate (K_2HPO_4) c.p.................	1.0 gm.
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) c.p................	0.5 gm.
Sodium chloride c.p...............................	0.1 gm.
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) c.p.....................	Trace
Glucose...	10 gm.
Casein..	10 gm.
Distilled water...................................	1000 cc.

Dissolve the casein in 80 cc. of 0.1N NaOH, then adjust to pH7.4 with 1N HCl.

Mix the other ingredients with the distilled water. Add the casein solution and mix thoroughly. Dispense into test tubes. Sterilize in an autoclave for 30 min. at 15 lb. pressure.

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* The medium, in dehydrated form, may be purchased from the Baltimore Biological Laboratory, 432 N. Calvert Street, Baltimore, Md.

42. *Winogradsky's Ammonium Sulfate Solution*

Ammonium sulfate c.p...............................	2 gm.
Dipotassium phosphate (K_2HPO_4) c.p..................	1 gm.
Magnesium sulfate ($MgSO_4·7H_2O$) c.p.................	0.5 gm.
Sodium chloride c.p..................................	2 gm.
Ferrous sulfate ($FeSO_4·7H_2O$) c.p.....................	0.4 gm.
Magnesium carbonate $(MgCO_3)_4Mg(OH)_2·5H_2O$ c.p......	10 gm.
Distilled water......................................	1000 cc.

Mix the ingredients with the distilled water. Dispense 100-cc. amounts into flasks. Stir often while dispensing to keep the magnesium carbonate in suspension. Sterilize in an autoclave for 30 min. at 15 lb. pressure.